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Biochemical and molecular characterisation of the dyslipidaemia in Portugal

Doutoramento em Biologia
Especialidade de Biologia de Sistemas

Cibelle Neiva Cavalcanti Mariano da Costa

Tese orientada por:
Doutora Mafalda Bourbon e Doutora Marília Antunes

Documento especialmente elaborado para a obtenção do grau de doutor

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Doutor Ricardo Jorge

“A mente humana não tem acesso à totalidade das causas dos fenómenos. A alma humana, porém, foi provida da necessidade de procurar as causas.”

Lev Tolstói

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ABBREVIATIONS

AACE	American Association of Clinical Endocrinologists
ABCG5/8	ATP-binding cassette sub-family G member 5/8
ACES	Health centres groups
ALB	Albumin
ALT	Alanine aminotransferase
ANGPTL3	Angiopoietin-like 3
apoA1	Apolipoprotein A1
apoB	Apolipoprotein B
APOC2	Apolipoprotein C2
APOE	Apolipoprotein E
AST	Aspartate aminotransferase
BMI	Body mass index
CI	Confidence intervals
CT	Colesterol total
CELSR2	Cadherin EGF LAG seven-pass G-type receptor 2
cDNA	Complementary deoxyribonucleic acid
CHD	Coronary heart disease
CS	Health centres
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DLCN	Dutch Lipid Clinic Network
DLCNS	Dutch Lipid Clinic Network score

DNA	Deoxyribonucleic acid
DRECE	*Dieta y Riesgo de Enfermedad Cardiovascular en España*
EAS	European Atherosclerosis Society
e_COR	*Estudo do Coração*
EPHF	Estudo Português de Hipercolesterolemia Familiar
ESC	European Society of Cardiology
ESH	European Society of Hypertension
FH	Familial Hyperchoelsterolaemia
FH/M-	Familial Hyperchoelsterolaemia mutation negative
FH/M+	Familial Hyperchoelsterolaemia mutation positive
GGT	Gamma-glutamyl transpeptidase
gnomAD	Genome Aggregation Database
GR	Genetic risk score
1000G	1000 genome
HDL-C	High-density lipoprotein cholesterol
HeFH	Heterozygous Familial Hypercholesterolaemia
HFE	Human hemochromatosis protein
HT	Hypertension
ID	Identification number
INE	Instituto Nacional de Estatística
INSA	National Institute of Health
IPAQ	Physical Activity Questionnaire
IQR	Interquartile range

LALD	Lysosomal Acid Lipase Deficiency
LIPA	Lisosomal acid lipase
LCAT	Lecithin-Cholesterol Acyltransferase
LDL-C	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein cholesterol receptor
Lp(a)	Lipoprotein(a)
LPL	Lipoprotein lipase
MAF	Minor allele frequency
MI	Myocardial infarction
MYLIP	Myosin regulatory light chain interacting protein
NGS	Next-generation sequencing
NHANES	National Health and Nutrition Examination Survey
NUTSII	Nomenclatura das Unidades Territoriais número 2
NYNRYN	NYN domain and retroviral integrase containing
P	Percentile
Pth	Percentile
pCVD	Premature cardiovascular disease
PHC	Primary health care
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type 9
RNU	National Register of users
SB	Simon Broome
SBP	Systolic blood pressure

SD	Standard deviation
SE	Standard error
SLC22A1	Solute sarrier family 22 member 1
sdLDL-C	Small, dense low-density lipoprotein cholesterol
SNP	Single nucleotide polymorphism
SORT1	Sortilin 1
ST3GAL4	ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 4
TC	Total cholesterol
TG	Triglycerides
WHO	World Health Organization
VUS	Variance of uncertain significance
UDR	Diagnosis and Reference Unit

ABSTRACT

Dyslipidaemia is one of the major modifiable independent risk factors for cardiovascular disease (CVD), with both genetic and environmental determinants. Although genetic risk factors are considered as non-modifiable, their CVD-associated risk can be prevented if early identified. The correct and early identification of dyslipidaemia is important for a better patient management and could definitely contribute to CVD prevention. This thesis intended the most complete characterisation of the dyslipidaemia in the Portuguese population, both biochemically and molecularly.

Reference values based on population-specific percentiles for lipid and lipoprotein biomarkers were provided for the first time in the Portuguese population, namely total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), apolipoprotein A1 (apoA1), apolipoprotein B (apoB), small, dense LDL-C (sdLDL-C), lipoprotein(a) [Lp(a)], as well apoB/apoA1 and sdLDL-C/LDL-C ratios, and non-HDL-C and remnant cholesterol. To our knowledge, the sdLDL-C percentiles were the first to be established in an European population. The percentiles were estimated through a rigorous methodology and compared with other population percentiles by a very visual and feasible method, showing relevant differences. These newly determined reference values for lipid biomarkers were then used to characterise the dyslipidaemia in our population, and can now be used in the clinic for a better patient care and management. More than cholesterol per se, our study highlighted apoB and sdLDL-C as important biomarkers to be used in dyslipidaemia evaluation. Individuals presenting extreme phenotypes were further investigated to assess possible monogenic causes, and three individuals were found to have familial hypercholesterolemia (FH), the most common genetic dyslipidaemia and one of the most common disorders that confer an increased cardiovascular risk. Finally, in an attempt to explore the causes for the FH phenotype, a polygenic risk score was validated for the first time in the Portuguese population. A total of 289 index cases were identified with monogenic FH and other causes for their dyslipidaemia, and also 100 were identified with polygenic hypercholesterolaemia, representing 53.21% of the cohort. From the monogenic causes, 91.35% have a mutation in *LDLR*, 4.84% in *APOB*, 1.04% in *PCSK9* and 2.08% had mutations in phenocopies genes (*LIPA*, *APOE*, *ALB*), suggesting that all those monogenic and polygenic causes should be always investigated for a better patient identification.

This study provided the most complete characterisation of the dyslipidaemia in the Portuguese population, and important evidences for dyslipidaemia evaluation has been

produced. The results obtained have application, not only for Portugal or a south European populations, but also might have an worldwide utility for the dyslipidaemia assessment. Together, the results obtained provide useful information on an important cardiovascular risk factor and should help to tackle and identify at risk situations that need urgent measures.

Keywords: Dyslipidaemia; Lipid reference values; Lipid biomarkers; Familial Hypercholesterolaemia; Polygenic hypercholesterolaemia.

RESUMO

As doenças cérebro-cardiovasculares (DCV), particularmente a doença coronária e o acidente vascular cerebral, são as principais causas de morbilidade e mortalidade a nível mundial. A incidência destas doenças tem vindo a aumentar nos países de baixo e médio rendimento, como resultado da modificação dos estilos de vida e do aumento da prevalência de fatores de risco cardiovascular. As formas mais comuns de DCV têm uma etiologia complexa, onde as interações entre os fatores genéticos e ambientais desempenham um papel importante. De entre os fatores de risco conhecidos para as doenças cardiovasculares, a dislipidemia, a hipertensão arterial, a diabetes, o sedentarismo, o excesso de peso/obesidade, a dieta inadequada e o tabagismo são fatores de risco modificáveis, e, portanto, passíveis de correção, o que pode minimizar situações de doença. Por outro lado, os fatores de risco genéticos não são modificáveis, mas se identificados precocemente, o risco associado pode ser minimizado, o que torna os estudos aprofundados sobre fatores de risco e suas causas (primárias como as genéticas, ou secundarias como os estilos de vida) uma prioridade na investigação da etiologia das DCV. De fato, no que concerne a dislipidemia, numerosas variantes genéticas (de raras a comuns) com efeitos significativos nos níveis plasmáticos de lípidos e lipoproteínas têm sido identificadas através das novas tecnologias de sequenciação do DNA e estudos de associação genómica (GWAS), entre outros. Uma vez que a dislipidemia é um dos principais fatores de risco cardiovascular, o conhecimento do perfil lipídico de uma população é de grande importância na implementação de intervenções preventivas específicas, com o objetivo de mudar as tendências da mortalidade cardiovascular. Assim, o objetivo principal deste presente estudo foi uma completa caracterização bioquímica e molecular da dislipidemia na população portuguesa.

Neste trabalho, numa primeira fase, determinaram-se intervalos de referência para os biomarcadores do metabolismo dos lípidos e lipoproteínas, tendo-se estabelecido percentis específicos para a população portuguesa, estimados através de *bootstrapping,* e tendo em consideração a distribuição populacional portuguesa de acordo com o sexo e a idade. Indivíduos sob terapia hipolipemiante, com diabetes, hipertiroidismo ou hipotireoidismo foram excluídos da análise de estimação dos percentis. Os percentis 5, 10, 25, 50, 75, 90 e 95 foram obtidos a partir de 866 indivíduos da população portuguesa (estudo e_COR) para doze biomarcadores: colesterol total (CT), colesterol da lipoproteína de baixa densidade (LDL-C), colesterol da lipoproteína de alta densidade (HDL-C), triglicéridos (TG), apolipoproteínas (apo) apoA1, apoB, colesterol das lipoproteínas de baixa densidade pequenas e densas (sdLDL-C), lipoproteína(a) [Lp(a)],

bem como para as razões apoB/apoA1 e sdLDL-C/LDL-C, colesterol não-HDL-C e colesterol das remanescentes. Os valores do percentil 50 para CT e LDL-C são semelhantes aos valores recomendados pela Sociedade Europeia de Cardiologia (ESC). O percentil 50 dos TG (83 mg/dL) é o mais discrepante dos valores recomendados (<150 mg/dL). Para a maioria dos restantes parâmetros, não há valores de referência consensuais para avaliação da dislipidemia. Os percentis estimados foram também comparados com os percentis de outras populações, através da construção de gráficos com os percentis de cada estudo, juntamente com os percentis estimados para a população portuguesa (e_COR) e os respetivos intervalos de confiança (95%). A comparação dos percentis entre o estudo e_COR e os usuários de cuidados de saúde primários em Portugal, e as populações espanhola e americanas apresentaram diferenças relevantes, principalmente nos valores de triglicéridos.

Posteriormente, a dislipidemia como fator de risco cardiovascular, foi avaliada em 1688 indivíduos do estudo e_COR utilizando como valores e intervalos de referência os percentis previamente estimados. Foi observada uma prevalência alta para a dislipidemia grave (acima do percentil 90) em relação aos diferentes biomarcadores, sendo os valores mais altos encontrados para o CT, apoB e LDL-C (16,22%, 16,02%, e 15,85%, respetivamente) com 22.90% dos participantes sob terapia hipolipemiante. A prevalência da hipercolesterolemia demonstrou ser maior em mulheres, ao contrário dos resultados para a prevalência da hipertrigliceridemia, os quais apresentaram-se maior nos homens. Também foi analisada a associação dos lípidos e lipoproteínas com outros fatores de risco não lipídicos, cujos resultados mostraram um perfil muito similar entre os biomarcadores apoB e sdLDL-C: o aumento dos valores de apoB e sdLDL-C aumentam no mesmo sentido dos valores de outros parâmetros lipídicos (CT, LDL-C, TG, não-HDL-C), bem como dos valores de índice de massa corporal, pressão arterial e quantidade de álcool, evidenciando assim a importância destes marcadores aterogénicos para além do colesterol. Foram também analisados, molecularmente, indivíduos nos extremos lipídicos com o objetivo de encontrar a causa do fenótipo, tendo sido identificados 3 indivíduos com uma alteração causadora de hipercolesterolemia familiar (FH), uma das hipercolesterolemias genéticas mais comuns.

Finalmente, e na tentativa de explorar as causas do fenótipo de FH, analisou-se a coorte do Estudo Português de FH (EPHF). Todos os resultados do estudo entre 1999 e 2016 foram avaliados tendo em conta tanto as causas monogénicas quanto as causas poligénicas. Validou-se para a população portuguesa o *score* de risco genético para o LDL-C, utilizando-se 6 polimorfismos de nucleótido único (SNPs) associados ao LDL-C *(CELSR2/SORT1*, rs629301; *APOB*, rs1367117; *ABCG5/8*, s4299376; *LDLR*, rs6511720;

e *APOE*, rs7412 e rs429358), previamente determinados num estudo do Reino Unido, e explorou-se a sua aplicabilidade na caracterização da hipercolesterolemia poligénica na coorte do EPHF. Por fim, comparou-se os resultados de diferentes critérios clínicos de FH na coorte de adultos, nomeadamente os critérios do Dutch Lipid Clinic (DLCN) e Simon Broome (SB). Ao todo, foram identificados com sucesso 39,53% (n=289) casos índex com dislipidemia monogénica, incluindo FH, disbetalipoproteinemia, sitosterolemia, deficiência em lipase ácida lisossómica e analbuminemia congénita, e ainda13,68% (n=100) com hipercolesterolemia poligénica (acima do percentil 75), ou seja, em 53,21% (n=389) foi possível identificar a causa da hipercolesterolemia. Se todas as variantes de significado incerto (VUS) previamente identificadas nos indivíduos (n=44; 6.02%) forem de fato patogénicas, o número total de indivíduos com causa monogénica aumentará para 45,55%. Dos restantes 298 doentes FH negativos, cerca de 9,39% (n=28) estavam abaixo do percentil 25, sugerindo outra potencial causa monogénica para a dislipidemia. Ao comparar os resultados entre os critérios DLCN e SB, não foram encontradas diferenças significativas. Embora o SB apresente uma ligeira melhoria na taxa de positividade, conclui-se que, sendo os resultados semelhantes, não há diferenças no poder discriminatório.

O presente estudo permitiu determinar os primeiros valores de referência de biomarcadores lipídicos na população portuguesa, com base nos percentis estimados para a população portuguesa. O percentil 50 é considerado ótimo e o intervalo entre os percentis 25 e 75 deve ser utilizado como valor de referência para cada parâmetro. Considerou-se que, acima do percentil 90 ou 95, ou abaixo do 10 ou 5 (HDL-C, ApoA1), são valores de alto e muito alto risco, respetivamente. Os percentis do sdLDL-C foram os primeiros a serem estabelecidos para uma população da Europa. Apresentamos também um método muito ilustrativo e viável para análise comparativa de valores de percentis. A prevalência dos diferentes biomarcadores do metabolismo lipídico também foi determinada e mostrou uma prevalência alta de dislipidemia grave, apesar da venda de estatinas ter aumentado exponencialmente nos últimos 10 anos. Ao contrário de outros estudos, a prevalência da hipercolesterolemia apresentou-se maior nas mulheres, provavelmente devido a utilização dos percentis específicos da população para sexo e idade, revelando que a dislipidemia pode estar subdiagnosticada neste género, e evidenciando a importância de valores de referência específicos para sexo e idade.

Os resultados demonstraram que a estimativa de valores de referência específicos da população é importante para a definição de “normalidade” e valores de risco. Estes percentis podem ser utilizados na prática clínica para identificação e controlo de doentes. Embora tenham sido encontrados valores altos para a dislipidemia, a mesma é um fator

de risco cardiovascular modificável, pelo que alterações nos estilos de vida e políticas de saúde devem ser implementadas para combater este fator de risco. Este estudo também preenche uma lacuna de mais de dez anos sem dados sobre a prevalência da dislipidemia na nossa população. A identificação de um problema de saúde pública é o primeiro passo para iniciar medidas preventivas.

As dislipidemias monogénicas estão associadas a um risco elevado de DCV *per se*, como a FH, enquanto que a maioria das dislipidemias leves a graves são devidas a causa poligénica, como resultado de várias alterações genéticas que podem interagir e ao mesmo tempo serem moduladas por fatores ambientais. Sendo assim a avaliação da etiologia da dislipidemia é de extrema importância para a prevenção cardiovascular. Tendo em conta que os doentes com FH podem ter a sua a expectativa de vida aumentada se forem identificados e tratados precocemente, a FH conjuntamente com outros fatores de risco cardiovascular devem ser avaliados na infância com o objetivo de reduzir o risco de DCV na idade adulta.

O presente estudo contribuiu para uma caracterização mais completa da dilispidemia na população portuguesa. Os resultados deste estudo podem servir de base para a implementação atempada de intervenções direcionadas para a prevenção de CVD.

Palavras-chave: Dislipidemia; Valores de referência lipídicos; Biomarcadores lipídicos; Hipercolesterolemia Familiar; Hipercolesterolemia poligénica.

Chapter 1

GENERAL INTRODUCTION

1. METABOLISM OF LIPIDS AND LIPOPROTEINS

1.1. Lipids and lipoproteins

Lipids are ubiquitous molecules found in animal and plant tissues, where they play important functional roles. There are five major groups of lipids, according to their chemical properties, namely fatty acids, sterol derivatives, glycerol esters, sphingosine, and terpenes (Naito, 1989), being this last one only found in plants (Ruzicka, 1953). Cholesterol and phospholipids are the major classes of lipids and an integral component of cellular membranes (Stein, 1986).

Cholesterol is a tetracyclic aromatic lipid that is a major constituent of the lipid bilayers of cellular membranes in eukaryotes, as well as of myelin sheath, and a precursor of steroid hormones, vitamin D3 and bile acids (Rifai et al., 1999). Cholesterol is one of the lipids with more clinical relevance and is produced in several types of tissues, being in humans mainly synthesized in liver. The cholesterol can be absorbed from diet into intestinal enterocytes in the jejunum, or synthesized in the liver from acetyl-CoA by a multistep process primarily begin with the conversion of acetyl-CoA to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA). Expression levels of HMG-CoA are negatively controlled by intracellular cholesterol, resulting in a powerful system of self-regulation (Goldstein et al., 1995). In physiological conditions, the major contributor to levels of cholesterol in circulation is *de novo* synthesis and not absorbed from diet (Dietschy et al., 1993; Brown and Goldstein, 1997; Liscum, 2002).

Other important class of lipids are the triglycerides (TG), which are formed by the derivation of glycerol-3-phosphate with a third fatty-acid chain in place of the phosphodiester-linked head group (Blassberg and Jacob, 2017). Unlike cholesterol and phospholipids, TG are not constituents of lipid bilayer membranes, but have an important energy-storage function. As a lipid, TG does not dissolve in the water phase, so TG hydrolysis reaction has to take place at the interface of the water and lipid phase. This reaction yields one molecule of glycerol and three molecules of fatty acid per mole of TG, which now can be dissolved in the lipid and water phase, respectively. With this way, fatty acid molecules can be captured by adipocytes for energy storage, or combined with albumin proteins in blood plasma, to be transported to muscle as energy source (Fredrickson et al., 1967). As cholesterol, TG can be absorbed from diet or synthesised *de novo* in the liver. However, in contrast to the cholesterol, the level of TG in circulation is mostly dependent of diet (Iqbal and Hussain, 2009; den Besten et al., 2013; Dash et al., 2015; Beilstein et al., 2016).

As lipids are a diverse group of organic compounds, insoluble in water and soluble in nonpolar solvents, their transportation in blood is accomplished through the use of specialized spherical particles called lipoproteins. Lipoproteins are special lipid-carrying molecules that act as a complex transport vehicle for fatty acids and cholesterol throughout the bloodstream. The surface of lipoproteins is composed by unesterified cholesterol (free cholesterol), phospholipids (e.g. lecithin) and sphingomyelin, and also by apolipoproteins. Cholesterol ester and TG are the nonpolar lipids that form the core of the lipoprotein particle (Fredrickson et al., 1967). There are four main types of apolipoproteins known as apolipoprotein A (apoA), B (apoB), C (apoC), and E (apoE) required for such transport, in order to help the solubilisation of hydrophobic lipids. They differ in their terminal residues, total amino acid content, and immunochemical behaviour (Fredrickson, 1974). These apolipoproteins function as cofactor, as lipid transfer proteins, and also as ligands for interaction with lipoprotein receptors in tissues (Rifai et al., 1999).

Lipoproteins vary in chemical structure and lipid and protein composition (Figure 1), being classified by several authors in the 50s and 60s (Gofman et al., 1952, 1954; Fredrickson et al., 1967). They include chylomicrons, very low-density lipoprotein (VLDL), intermediate low-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) (Table 1). The VLDL, IDL, LDL and HDL are always presented in normal plasma, while chylomicrons are transiently following a fat-containing meal and are metabolised rapidly (usually removed from plasma after 12 hours, approximately) (Bachorik, 2010). Further studies demonstrated that high levels of LDL particles in the blood were associated with premature cardiovascular disease (CVD) (Kannel et al., 1979; Campos et al., 1992), while high HDL levels had a protective effect (Gordon et al., 1977). In fact, HDL particles help to remove cholesterol from tissues, delivering it back to the liver. On the other hand, LDL particles can accumulate in the intimal sub-layer of arteries, by their propensity to bind to connective tissue (Daniels et al., 2009). This is why the cholesterol from HDL is commonly called “good cholesterol” and the cholesterol from LDL is called “bad cholesterol”.

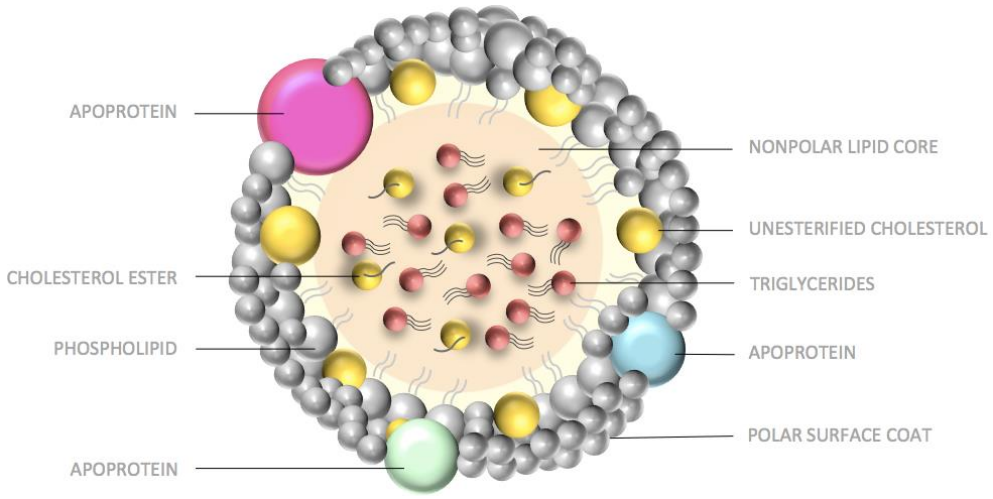

Figure 1 – Lipoprotein structure. The lipoproteins are spherical particles containing a central hydrophobic core of nonpolar lipids, primarily cholesterol esters and triglycerides. A hydrophilic membrane containing phospholipids, free cholesterol, and apolipoproteins surrounds the hydrophobic core.

Table 1 – Characteristics of human lipoproteins present in plasma (Rifai et al., 1999).

Variable	Chylomicron	VLDL	IDL	LDL	HDL	Lp(a)
Density, g/mL	<0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.210	1.040-1.130
Molecular weight, Da	0.4-30 x 10^9	5-10 x 10^6	3.9-4.8 x 10^6	2.75-10 x 10^6	1.8-3.6 x 10^5	2.9-3.7 x 10^6
Diameter, nm	>70	25-75	22-24	19-23	4-10	25-30
Lipid:protein ratio	99:1	90:10	85:15	80:20	50:50	75:25-64:34

VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Lp(a), lipoprotein(a).

1.2. Lipids and lipoproteins metabolism

Cholesterol molecules can derived from diet (exogenous) or through *de novo* synthesis (endogenous pathway). As a major component of cellular membranes, cholesterol is transported between organelles by intracellular trafficking processes. In the exogenous pathway, after absorption, TG and cholesterol are re-esterified in the intestinal mucosal cells and then coupled with various apoproteins, phospholipids, and unesterified cholesterol into lipoprotein particles called chylomicrons. These TG rich particles are then hydrolysed in circulation by the lipoprotein lipase (LPL), resulting in hydrophilic fatty acids that then pass through endothelial cells (capillary) to reach adipocytes (storage) and skeletal muscle cells (oxidation). After removal of the TG core, remnant chylomicron particles are formed, which are high in cholesterol esters and characterised by the presence of apoB, apoC3, and apoE. These remnants are cleared from the circulation by binding of their apoE to a receptor present only on the surface of hepatic cells.

Subsequently, these bound remnants are taken by the hepatocytes trough endocytosis and then catabolised by lysosomes. This process results in cholesterol release, which is then either converted into bile acids, excreted in bile, or incorporated into VLDL. In the endogenous pathway, TG are synthesized in the liver utilising free fatty acids and carbohydrates as substrates. They are secreted into the circulation in the core of VLDL. As these lipoproteins undergo TG hydrolysis, they become increasingly dense and cholesterol rich (IDL) by a subsequent interaction of the VLDL particles with LPL and liberation of free fatty acids. Some of these remnant particles are removed from the circulation, while the rest undergo modifications with detachment of the remaining TG and its substitution by cholesterol esters and removal of almost all apolipoproteins (except apoB), resulting in LDL particles even richer in cholesterol. At the same time, in the reverse transport of cholesterol, liver and small intestine secrete HDL particles that interacts with lipids and proteins released during the catabolism of TG-rich lipoproteins, resulting in a production of cholesteryl esters from the action of lecithin–cholesterol acyltransferase (LCAT). These cholesteryl esters are in turn transferred to VLDL and subsequently LDL. This process ends in the transfer of cholesterol through LDL to peripheral cells and its return to the liver through HDL, thus preventing excessive accumulation of cholesterol in the body. These interactions function as a system to maintain the cholesterol homeostasis (Figure 2).

1.2.1. Exogenous lipoproteins pathway

The exogenous lipoprotein pathway starts in the intestine. Dietary TG and cholesterol are absorbed by the enterocytes and packaged into chylomicrons. The uptake of cholesterol is facilitated by the Niemann-Pick C-like 1 protein (NPC1L1). This absorption by enterocytes can be selectively inhibited by a drug called ezetimibe, by inhibiting the NPC1L1 (Garcia-Calvo et al., 2005). Once in the enterocytes, the cholesterol may be transported back into the intestinal lumen, a process mediated by ATP-binding cassette sub-family G member 5 and 8 (ABCG5 and ABCG8): they form a complex that promotes secretion of cholesterol back into the intestinal lumen to be excreted through faeces (Daniels et al., 2009). The chylomicrons formation occurs in the endoplasmic reticulum and requires the synthesis of apoB48 by intestinal cells (Gordon et al., 1995). The composition and size of the chylomicrons formed in the intestine are dependent on the amount of fat ingested and absorbed by the intestine, as well as by the type of fat absorbed, so increased fat absorption results in larger chylomicrons.

About 90% of chylomicrons are TG, while 2% are proteins (apoB48, apoa1 and apoA4) (Rifai et al., 1999) and the remaining is cholesterol. The metabolism of the TG

carried in the chylomicrons results in a decrease in the size of these particles, leading to the formation of chylomicron remnants, which are enriched in cholesterol esters and acquire the apoE and apoC protein, a cofactor of the LPL (Havel and Kane, 1995). LPL is the enzyme that catalyses the TG hydrolysis in free fatty acids and glycerol (process called lipolysis) in circulation, which could be used by adipocytes as energy storage, or combined with albumin proteins in blood plasma to be transported to muscle as energy source (Fredrickson et al., 1967). During lipolysis, chylomicrons also decrease in size phospholipids and apolipoproteins (apoA and apoC) on the surface, which are transferred to other lipoproteins, mainly HDL. This transfer of apoC2 from chylomicrons to HDL particles decreases the ability of LPL to further breakdown TG (Goldberg, 1996). Thus, these chylomicron remnants are cleared from the circulation by the liver. The apoE on the chylomicron remnants binds to the LDL receptor (LDLR) and other hepatic receptors to be taken up by the hepatocytes (by endocytosis) (Mahley and Ji, 1999; Heeren et al., 2005). TG and cholesterol are hydrolysed in lysosomes of the hepatocytes. An unknown amount of cholesterol are transported back to the liver and form bile acids, which may then be excreted, can also be incorporated by lipoproteins synthesised *de novo*, or it can be esterified and stored by hepatocytes (Fredrickson et al., 1967; Ikonen, 2008).

1.2.2. Endogenous lipoproteins pathway

The TG can be synthesised from fat acid and carbohydrates by hepatocytes in the liver. As previously mentioned, cholesterol can be also synthesised *de novo*: when cholesterol is not sufficient, the transcription of the HMG-CoA enzyme is activated and cholesterol is synthesised by hepatocytes (Goldstein et al., 1995). TG and cholesterol are then packaged with the apoB100 in the endoplasmic reticulum of hepatocytes, forming the VLDL (Breslow, 1989). These VLDL goes to the Golgi apparatus by exocytosis to be delivered to the blood circulation.

In contrast to the chylomicrons, VLDL contains about 55% of TG (Bachorik, 2010) and 10% of apolipoproteins (including apoC2 and apoE, but mainly apoB100) (Breslow, 1989). The TG in VLDL are hydrolysed by LPL resulting in the formation of IDL, which can be cleared from plasma by hepatic LDLR. These receptors have high affinity to the apoE, so the clearance of ILDL from blood is very fast (Goldstein et al., 1995). Compared to the chylomicrons, the mean residence time of TG of VLDL is shorter (5 to 10 minutes *versus* 15 to 60 minutes), probably due to the small size of VLDL (Havel and Kane, 1995). Further lipolysis of the IDL particles remained in plasma occurs, forming even smaller particles, the LDL. Meanwhile, the excess of phospholipids, free

cholesterol and apolipoproteins are transferred to HDL particles or return to the blood circulation to form new particles.

LDL is a cholesterol-rich particle with apoB100 on its surface, since these apolipoproteins are the only ones not transferred during lipolysis. These apoB are recognised by hepatic and extra hepatic LDLR, and removed from plasma (approximately 70% is cleared via LDLR), occurring most of this process in the liver (Breslow, 1989). The LDL particles usually have higher residence time in plasma (72 hours, approximately) than VLDL (Havel and Kane, 1995), as mentioned before probably because of the high affinity for the LDLR of the apoE in VLDL. In this sense, it is important to note that the greater the VLDL clearance from plasma, the lower the fraction converted to LDL.

As mentioned before, once HMG-CoA enzyme is activated leading to a cholesterol synthesis by hepatocytes, inhibitors of the HMG-CoA enzyme have thus emerged as important pharmacologic treatments (called statins) for patients whose elevated cholesterol levels are refractory to dietary control. This drug has effect on intracellular cholesterol levels reduction, leading to a over-regulating of the expression of the *LDLR* gene, which results in an increased removal of the LDL-C from blood (Simes et al., 2002; Marks et al., 2003).

1.2.3. Low-density lipoprotein receptor pathway

The LDL particles are removed from plasma via LDLR through apoB100 on the surface of LDL (Figure 6). The process occurs as the following: LDLR internalises the complex LDL particle-LDLR into a clathrin vesicles. These clathrin vesicles are further fused to form endosomes. After endocytosis, occurs the disassociation of LDL from the LDLR by acidic conditions of the endosome (by lowering the pH, a dissociation of the complex within the endosome occurs) (Rader et al., 2003), leading to the LDLR recycling back to the cell membrane (Havel and Kane, 1995). LDL particles migrate to a lysosome, where apoB100 is degraded to amino acids and the cholesteryl esters hydrolysed by lysosomal acid lipase (LIPA) into free cholesterol, to be further incorporated into cell membranes, or used into bile acids, or synthesis of steroids (Liscum and Underwood, 1995; Rifai et al., 1999). In this process, there is a protein called propotein convertase subtilisin/kexin type 9 (PCSK9) that is involved in the regulation of the LDLR protein degradation, preventing it from being recycled to the cell surface: when *PCSK9* binds to LDLR on its cell surface, the LDLR-LDL-PCSK9 complexes are internalised into clathrin vesicles at endosome, where by pH lowering the binding is enhanced, preventing the LDLR-LDL-PCSK9 complexes dissociation and LDLR recycling, and leading to the LDLR degradation in the lysosome (Cunningham et al., 2007).

The remaining LDL not cleared from plasma is recognized by the scavenger receptors of cells, such as macrophages. These receptors on macrophages only recognises modified LDL particles (e.g. due to oxidation process) (Rifai et al., 1999), which are removed by them leading to the foam cell formation involved in the atherogenesis process (Tabas et al., 2015).

1.2.4. The reverse cholesterol transport pathway

This process allows the cholesterol that cannot be metabolised by peripheral tissues to move from these tissues back to the liver for excretion. This pathway involves HDL discoid particles (HDL_2), containing apoA1, apoE and phospholipids. In order to form mature large spherical HDL particles with a core of cholesterol esters, the free cholesterol transferred from cells to the surface of HDL particles must be esterified. This cholesterol is esterified in plasma by the LCAT enzyme, using the apoA1 as a cofactor (Breslow, 1989). The HDL size depends of the quantity of the esterified cholesterol and also of the LCAT activity (Rifai et al., 1999). The HDL_3 are the smallest particles that by the LCAT and LPL action enlarge HDL_3 to HDL_2, containing more apolipoproteins. These HDL_2 are further converted again to HDL_3 by the action of the cholesteryl ester transfer protein (CETP) and hepatic lipase C (LIPC). The cholesterol ester in HDL can be now transferred via CETP to the apo B-containing lipoproteins, VLDL, IDL and LDL, allowing the uptake of this cholesterol ester by the LDLR pathway in the liver (and the subsequent elimination of cholesterol from the body), or the apoE present in HDL particles can be recognised by the LDLR in the liver (Breslow, 1989).

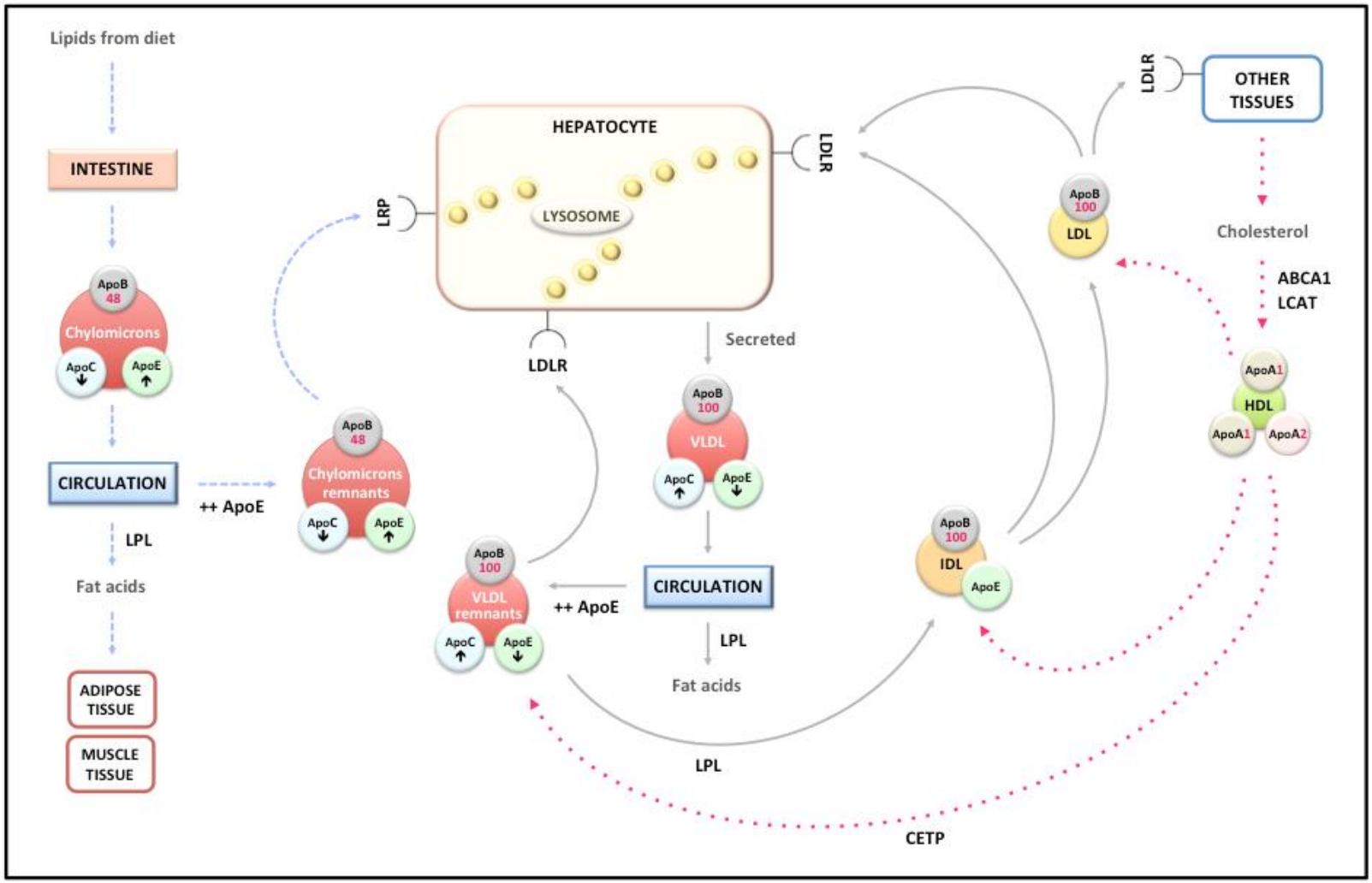

Figure 2 – Metabolism of the lipids and lipoproteins. Exogenous pathway is represented by the blue connector (dotted), endogenous pathway is represented by the grey connector (solid), and the reverse cholesterol transport pathway is represented by the pink connector (dotted point). Represented pathways are described in the section Metabolism of lipids and lipoproteins of this present study. ApoB, apolipoprotein B; ApoC, apolipoprotein C; ApoE,

apolipoprotein E; LPL, lipoprotein lipase; LRP, low density lipoprotein receptor-related protein; LDLR, low-density lipoprotein receptor; VLDL, very-low-density lipoprotein; ABCA1, ATP-binding cassette transporter; LCAT, lecithin–cholesterol acyltransferase; ApoA1, apolipoprotein A1; ApoA2, apolipoprotein A2.

2. CONSTITUENTS OF THE PLASMA LIPID PROFILE

The relationships between plasma lipids and lipoproteins and the risk of atherosclerosis development have been observed in human population studies for many years (Gotto et al., 1977; Regnström et al., 1992; Nordin Fredrikson et al., 2003; Marcovina and Packard, 2006; Bertoia et al., 2013). As several studies have demonstrated that interventions that target plasma lipids and lipoproteins have the potential to reduce the coronary heart disease (CHD) (Anderson, 2016; Emdin et al., 2016; Fitzgerald et al., 2017; Lloyd-Jones et al., 2017; Robinson et al., 2017; Stein et al., 2017), levels of cholesterol and related lipids circulating in plasma are considered as important biomarkers clinically used to predict the risk of a CVD event.

A standard lipid profile includes measurements of plasma or serum concentrations of total cholesterol (TC), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), and TG (Figure 3). These biomarkers can be supplemented with remnant cholesterol and non-HDL-C without additional cost. As non-HDL-C, apoB and apoA1 can be also used as atherogenic risk biomarkers, but these biochemical determinations can have additional costs. Finally, lipoprotein(a) [(Lp(a)] is other important additional measurement to be taking into account in the CVD risk prediction (Perk et al., 2012; Catapano et al., 2016).

2.1. Total cholesterol

The TC is a total amount of cholesterol in plasma, either from chylomicrons (nonfasting state), VLDL, IDL, LDL, HDL or Lp(a), and can be measured directly. Is a very common and recommended lipid parameter to be evaluated in dyslipidaemia screening. However, in some cases TC may be misleading, since HDL-C and TG can also contribute significantly to TC and would overestimate the risk for CVD if determined from TC alone. For example, women usually have high levels of TC than men due to higher HDL-C (Carroll *et al.*, 2012). On the other hand, individuals with diabetes or high TG who often have low HDL-C, have higher levels of TC (Talayero and Sacks, 2011). Therefore, LDL-C and HDL-C should be analysed for a more accurate evaluation. European guidelines (EAS/ESC) (Catapano et al., 2016) recommended TC levels below 190 mg/dL. Even so, in patients with familial hyperlipidaemia (e.g. Familial Hypercholesterolaemia), TC above 290 mg/dL should receive special attention, once these patients are always at high risk of CVD development (Marks et al., 2003).

2.2. Low-density lipoprotein cholesterol

The LDL-C is the cholesterol from LDL particles. These LDL particles are the main carriers of plasma cholesterol and was the first of the apoB-containing lipoproteins to be recognised as atherogenic (Saxena and Goldberg, 1994). In particular, LDL-C has considerable notoriety for its causative role in atherosclerosis, the leading cause of death and disability around the world. When intracellular cholesterol homeostasis is compromised, they can accumulate into artery walls and lead to atherosclerotic plaque development and subsequent CHD. As LDL particles are small, they can enter the vascular wall and persist in the circulation, being susceptible to oxidation and subsequently inflammation process involved in the atherogenesis process (Knott et al., 1986; Carr et al., 2000).

LDL-C is the primary treatment target for over 30 years and can either be measured directly or calculated by the Friedwald equation (if TG are below 400 mg/dL): TC minus HDL-C minus TG/5 (Friedewald et al., 1972). Despite calculated LDL-C be unreliable when are obtained from nonfasting samples, is still widely used. There are several studies demonstrating discrepancies between methods (Scharnagl et al., 2001; Sahu et al., 2005; Sibal *et al.*, 2010; Gupta et al., 2012; Martin et al., 2013; Anwar et al., 2014; Kannan et al., 2014), but is a valid estimation according to the European guidelines (Catapano et al., 2016). In fact, recent studies have been suggested that nonfasting lipid profiles have no negative implications for prognostic, diagnostic, or therapeutic options in CVD prevention (Nordestgaard et al., 2016; Nordestgaard, 2017). Additionally, even direct methods should be used with caution in patients with high TG levels, since they may underestimate low levels of LDL-C (Langlois et al., 2014). In these circumstances, non-HDL-C or apoB should be considered and used as secondary targets. ESC/EAS guidelines (Catapano et al., 2016) recommended LDL-C levels below 115 mg/dL. Individuals presenting LDL-C above 190 mg/dL are at high risk of CVD.

2.2.1. Small, dense low-density lipoprotein cholesterol

Human LDL particles can vary in density from 1.019 to 1.063 g ml^{-1}, approximately, containing about 50% of free and esterified cholesterol, 25% proteins, 20% phospholipids, and 5% TG. More than 90% of the LDL mass is apoB100, but each particle contains only one molecule of apoB (Havel et al., 1955; McNamara et al., 1987; Rajman et al., 1999). Importantly, LDL particles are very heterogeneous, varying in size, density, and composition, which can also vary among individuals. In 1988, Austin and colleagues (Austin et al., 1988) suggested two major patterns of LDL profile, pattern A with a particle

diameter of 25.5 nm or greater (density below the 1.038 gmL^{-1}), and pattern B with particle diameter less than 25.5 nm (density above the 1.038 gmL^{-1}). The biochemical processes that underlie the formation of such distinct subfractions are not completely understood, but several studies have demonstrated that pattern B, the small, dense LDL particles (sdLDL) is associated with CHD. The formation of these sdLDL particles seems to be related to the TG content, where VLDL particles gives rise to smaller LDL particles (cholesterol-rich) via lipolysis (Rajman et al., 1994). So, the removal of TG from the core of VLDL by LPL results in IDL particles that can be metabolised by the LPL and LIPC to LDL particles, leading to a continued particle size reduction: exchange core lipid with VLDL and chylomicrons to become TG rich and hence susceptible to the action of LIPC. The enzyme removes lipid from the particle and releases LDL that is smaller and denser than normal (Rajman et al., 1994; Packard C, 1996). In fact, epidemiological studies shown that the predominance of this particles is generally associated with high levels of TG and low levels of HDL-C (McNamara et al., 1987). The reason for the association of these sdLDL particles with CHD is explained by their susceptibility in the causal role of atherosclerosis process. They stimulate the superoxide production and consequently oxidation of LDL (Cooke and Tsao, 1994; Pritchard et al., 1995), they are smaller than other LDL particles subtraction, thus sdLDL more effectively enter in arterial intima, they are also more susceptible to the oxidation process (Steinberg et al., 1989; de Graaf et al., 1991; Dejager et al., 1993; Tribble, 1995), and finally they have lower affinity to the LDLR compared with larger LDL particles, resulting in reduced hepatic clearance and longer time in plasma (Nigon et al., 1991; Chen et al., 1994).

The sdLDL measurement is only possible through ultracentrifugation or polyacrylamide gel electrophoresis (Rajman et al., 1999), but immunoturbidimetric assays are now available to selectively determine the cholesterol content in those particles (sdLDL-C) in a simple and feasible way (Tsai et al., 2014). Despite all these evidences, the ESC/EAS guidelines (Catapano et al., 2016) did not recommended this lipid biomarker for the dyslipidaemia screening, so there are no recommended sdLDL-C values.

2.3. High-density lipoprotein cholesterol

The HDL-C is the cholesterol content of HDL particles. HDL particles carriers about 20% of the circulating cholesterol and is composed primarily of apoA1 and lecithin (Bachorik, 2010). While low levels of HDL-C have been considered as independent CVD risk factor, high levels have not been found as associated with protection against atherosclerosis (Haase et al., 2010; Triglyceride Coronary Disease Genetics Consortium and Emerging Risk Factors Collaboration et al., 2010; Voight et al., 2012;

Holmes et al., 2015). Even so, HDL quality has been suggested more relevant than HDL-C. Once HDL is highly heterogeneous, with two major subtractions (HDL_2 and HDL_3) that can be identified on the basis of density, size, charge, and protein composition, the concept that certain subtractions of HDL may be better predictors of CVD risk is reasonable (Khera et al., 2011; Kontush and Chapman, 2012; Li et al., 2013; Rohatgi et al., 2014). Several studies have demonstrated that higher HDL-C levels are not always associated with improved cardiovascular outcomes, showing that individuals with CHD have more dysfunctional HDL particles than health individuals (Andersen et al., 2003; de Goma et al., 2008; van der Steeg et al., 2008; Navab et al., 2011). In fact, there are many molecular mechanisms through which HDL may become dysfunctional, including changes in protein composition, change in antioxidant activity, enzymatic modification of constituent HDL proteins and lipids, oxidation, and others (Kones, 2011). For instance, Quispe and colleagues (Quispe et al., 2015) found that very high HDL-C levels were associated with a higher HDL_2-C/HDL_3-C ratio, while van der Steeg et al. (van der Steeg et al., 2008) showed that when apoA1 and apoB are kept constant, HDL-C and HDL particle size might confer risk at very high values, so HDL_3 may have greater potential for modulating antiatherogenic effects compared with HDL_2 (Camont et al., 2013).

Notwithstanding, epidemiological studies suggested that HDL-C levels below 40 mg/dL in men and below 48 mg/dL in women are associated with increased CVD risk, so above these cut-offs are the recommended values from ESC/EAS guidelines (Catapano et al., 2016). As LDL-C, HDL-C can be measured directly, but in patients with high levels of TG this measurement should be used with caution.

2.4. Triglycerides

The TG is other major lipid biomarker for dyslipidaemia evaluation. They can be determined directly with very low rate of error. All lipoproteins have TG in its contents, but in different concentrations. Chylomicrons, VLDL and IDL are the main carriers of TG in plasma and are considered as atherogenic particles. When they persist in the circulation too long, their cholesterol content increases and TG-rich particles become smaller and more depleted of TG (Bachorik, 2010). TG and remnant lipoproteins (chylomicrons remnants and VLDL remnants) have been associated with increased CVD risk (Hokanson and Austin, 1996; Sarwar et al., 2007). Despite chylomicrons be too large to enter the arterial intima, remnant lipoproteins can enter the arterial intima just like LDL particles, so another mechanism suggested is that once in arterial intima, the LPL activity as well as the liberation of free fat acids and other molecules can lead to injury and inflammation

processes (Nordestgaard and Varbo, 2014).

Other mechanisms fro the role of TG in CVD have been suggested, but they are not completely understood. Indeed, their role as independent risk factor was strongly suggested, whereby remnant cholesterol is also refereed as an important biomarker (Varbo et al., 2013a, 2013b; Nordestgaard and Varbo, 2014). Thus, high TG levels can be considered as a biomarker for atherogenic lipoproteins, particularly important in patients with insulin resistant syndromes such as type 2 diabetes mellitus and metabolic syndrome, who commonly present with combined dyslipidaemia (Ford et al., 2002; Alexander et al., 2003). Remnant cholesterol can be easily calculated as TC minus LDL-C minus HDL-C in fasting (cholesterol content of VLDL and IDL) or nonfasting (cholesterol content of VLDL and IDL together with chylomicron remnants) states (Nordestgaard et al., 2016), or can be measured directly. The TG recommended value by ESC/EAS guidelines (Catapano et al., 2016) is below 150 mg/dL, while for remnants there are no recommended values yet.

2.5. Non-high-density lipoprotein cholesterol

The non-HDL-C represents the cholesterol concentration transported by all atherogenic lipoproteins, being a measure of the mass of cholesterol within VLDL, IDL, LDL, and Lp(a) (together with chylomicron remnants in nonfasting state). Several studies have been shown this lipid biomarker as a better predictor of CVD risk than LDL-C, and that is clinically equivalent to the apoB (Ramjee et al., 2011; Boekholdt et al., 2012; Emerging Risk Factors Collaboration et al., 2012; Robinson et al., 2012; Pencina et al., 2015). However, results are inconclusive, whereby LDL-C is still recommended as primary treatment target, and non-HDL-C recommended as a secondary target (Catapano et al., 2016). Indeed, as previously mentioned, non-HDL-C can overcome some limitations of FriedWald's equation and direct LDL-C estimations, especially in cases of patient's high levels of TG and low levels of LDL-C. In addition, as also mentioned in the previous section, the strong results supporting the VLDL, IDL and remnants particles as causative factors in atherogenesis, suggest this lipid parameter as a good biomarker for the dyslipidaemia screening.

Non-HDL-C is easily calculated from TC minus HDL-C, which make it very cost-effective. ESC/EAS guidelines (Catapano et al., 2016) recommended values below 145 mg/dL for patients at low to moderate risk, values below 130 mg/dL for patients at high risk, and values below 100 mg/dL for patients at very high risk for CVD.

2.6. Apolipoproteins

The apoB and apoA1 measurements can be used as alternatives to non-HDL-C and HDL-C, respectively, but despite be easily from a technical point of view, these determinations come at extra cost (Hegele et al., 2014; Catapano et al., 2016). Even so, they not require fasting conditions and are not volatile under high TG levels, allowing more accurate results. When available, the apoB/apoA1 ratio can be calculated and used for CVD risk estimation. However, they are not considered for diagnosis or treatment targets.

There are no recommended values by ESC/EAS guidelines, but important prospective studies (AMORIS and INTERHEART studies) (Walldius et al., 2001; Yusuf et al., 2004) suggested apoB/apoA1 ratio values below 0.7 mg/dL in men and 0.6 mg/dL in women as low risk, values between 0.7 and 0.9 mg/dL in men and 0.6 to 0.8 mg/dL in women as moderate risk, and values above 0.9 mg/dL in men and 0.8 mg/dL in women as high risk for CVD development.

2.6.1. Apolipoprotein B

The apoB is the major apolipoprotein of all VLDL, IDL and LDL lipoproteins. This apolipoprotein play an important role in the lipid metabolism, because it is essential for the binding of LDL particles to the LDLR, allowing the LDL-C clearance (Havel and Kane, 1995; Rifai et al., 1999). Once each lipoprotein particle contains only one molecule of apoB, this lipid biomarker is a good estimator of the number of all atherogenic particles in plasma (Rajman et al., 1994, 1999).

It is important to note that even in cases of low LDL-C, high apoB and sdLDL-C concentrations might be present. This happens because small LDL particles contains less cholesterol than large particles; as previously mentioned, more than 90% of apoB are in the LDL particles, so the apoB level might provide satisfactory estimation of the LDL particles, as well as of the sdLDL-C (Sniderman et al., 2014). In this sense, is reasonable to consider this lipid biomarker as a potential target for dyslipidaemia evaluation, specially because CVD risk is apparently greater in younger individuals exposed to CVD risk factors (Sniderman et al., 2016). Also, in 2008 Sniderman (Sniderman, 2008) showed that the decrease in apoB was 79% of the decrease in LDL-C and 84% of the decrease in non-HDL-C after treatment with statins, showing be effective in the apoB levels reduction.

However, apoB is only recommended as secondary target by ESC/EAS guidelines (Catapano et al., 2016). This is justified by the lack of evidence of their benefit beyond non-HDL-C or even beyond traditional lipid biomarkers in individuals with diabetes (Taskinen et al., 2010). When apoB is available, recommended levels are apoB below

100 mg/dL in those individuals at high risk and below 80 mg/dL at very high risk for CVD development. Still, the American Association of Clinical Endocrinologists guidelines (AACE/ACE) (Jellinger et al., 2016) recommended apoB levels below 90 mg/dL, considering values above that cut-off as moderate CVD risk.

2.6.2. Apolipoprotein A1

The apoA1 is the major apolipoprotein found in HDL particles. Physically, apoA1 occupies about 85% of the surface of HDL (Jones et al., 2010; Huang et al., 2011; Zhang et al., 2013). There are one to five molecules for each HDL particle, approximately, which may reflect in a not very precise estimation of the HDL-C concentration. As previously described, this apolipoprotein has an important role in reverse cholesterol transport, where enables the efflux of excess of cholesterol from cells and the transfer back to the HDL particles (Marcovina and Packard, 2006; Walldius and Jungner, 2006). An additional antiatherogenic property of apoA1 is the anti-inflammatory and antioxidant effects (Shah et al., 2001; Schlitt et al., 2005; Barter and Rye, 2006; Navab et al., 2011). As epidemiological studies have demonstrated the apoA1 as an important CVD risk predictor, ESC/EAS guidelines (Catapano et al., 2016) recommended values above 120 mg/dL for men and above 140 mg/dL for women.

2.7. Lipoprotein(a)

The Lp(a) is a plasma lipoprotein composed by a cholesterol-rich LDL particle and one molecule of apoB100 covalently linked to a apolipoprotein(a) (Figure 3). About 30% of total Lp(a) mass is cholesterol. This apolipoprotein(a) is structurally homologous to plasminogen, which can increase the CVD risk via prothrombotic/anti-fibrinolytic effects (Utermann, 2001). Lp(a) is considered as risk factors for many years, with several studies showing the association between Lp(a) concentration and risk of CVD. The plasma levels of Lp(a) are mainly related to the polymorphism in the apolipoprotein(a) gene. The apolipoprotein(a) contains ten distinct types of plasminogen kringle 4-like repeats, homologous regions to the kringle 5 and a protease. Furthermore, contains a different number of repeated kringle 4 type 2 domain (can vary from 2 to more than 40), giving rise to different-sized apolipoprotein(a) isoforms (Utermann, 2001; Nordestgaard et al., 2010). In fact, there is a large variation in plasma Lp(a) levels that could be explained by this genetic variation (Sandholzer et al., 1991; Kamstrup et al., 2008, 2009; Tsimikas, 2017) that might be involved in the pathophysiology of CHD.

Lp(a) measurement is not recommended by ESC/EAS guidelines (Catapano et al., 2016) in the general dyslipidaemia evaluation, but should be considered

in individuals with high CVD risk. The Lp(a) values considered as risk are above 50 mg/dL, which corresponds to the 80th percentile in a European population (Copenhagen general Population) (Nordestgaard et al., 2010). Despite Lp(a) measurement be very stable over time (variation is lower than 10%), there is no standardization between assays, which make it difficult the results interpretation and evaluation by the clinicians (apart from being expensive) (Marcovina et al., 2003). Importantly, besides effect on CVD events has not been shown, the Lp(a) levels in plasma can be reduced up to 30% with the lipid-lowering therapy PCSK9 inhibitors and also with nicotinic acid (Seed et al., 1993; Robinson et al., 2015; Sabatine et al., 2015), and can be reduced up to 80% with antisense drugs targeting the Lp(a) gene (Tsimikas, 2016). If we have into account that Lp(a) contains a high percentage of cholesterol, it is reasonable to think in this lipid biomarker as a potential target.

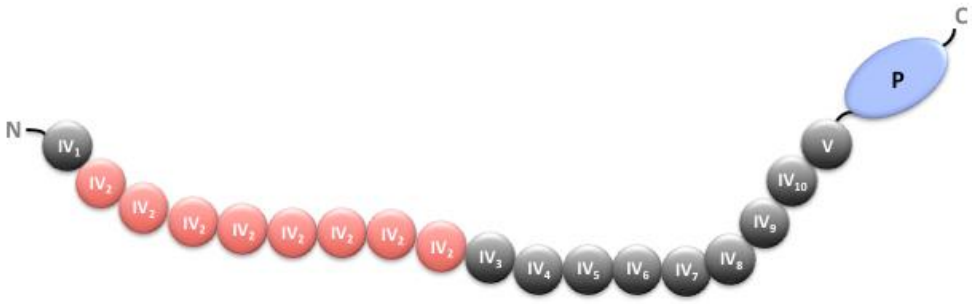

Figure 3 – Apolipoprotein(a). Apolipoprotein(a) contains ten distinct types of plasminogen kringle 4-like repeats (IV1 to IV10), homologous regions to the kringle 5 (V), and a protease (P). Also contains a different number of repeated kringle 4 type 2 domain that can vary from 2 to more than 40 (IV2), giving rise to a different-sized apolipoprotein(a) isoforms. Inhibition of the N-terminal (N) glycosylation can reduce the apolipoprotein(a) secretion, while the protease inhibitors can increase secretion. C-terminal (C) is involved in the protein secretion by reducing the pre-secretion degradation (Hoover-Plow and Huang, 2013).

3. REFERENCE VALUES AND INTERVALS FOR LIPID AND LIPOPROTEIN BIOMARKERS

Reference values of plasma biomarkers are statistically derived numbers from a reference population, Ideally, the individuals should be selected from a reference population, ideally using specific criteria (age, gender, race, etc.) and including exclusion criteria (e.g. tobacco use, medications, etc.) (Horowitz, 2008). Well-established biomarker reference ranges provide a baseline to assess the clinical status of an individual and/or population. These biomarkers are commonly used in basic and clinical research and in community assessments, such as policymakers use population-level biomarkers for

screening, surveillance, and monitoring/evaluation of interventions. Clinicians use biomarkers mainly for diagnosis, prognosis, and treatment (Schulte, 2005).

A reference value for lipids and lipoproteins is a specified quantitative measure used to assess the presence or absence of a health-related condition. They generally reflect the underlying health status of the population and are required for translating, stratifying, and reliably distinguishing the spectrum of results that represents a continuum from deficiency to excess obtained from a biomarker analysis (Klee, 2004). The reference values for lipids and lipoproteins are usually recommended values from clinical practice guidelines that were established based on the risk for CVD development (Catapano et al., 2016). Because of genetic, behavioural and environmental differences among different populations, variation in these reference values should be expected. As a European population, the reference/recommended values usually used in Portugal are from European guidelines, otherwise American guidelines are consulted. But it is important to note that reference/recommended values for some lipids and lipoproteins biomarkers are still missing. Thus, it is reasonable to think in the importance of the population-specific reference values estimation.

There are different ways to establish reference values, and percentiles estimation is one of them. Lipids and lipoproteins percentiles were never determined for the Portuguese population, although they have been for specific subpopulations as part of different studies. In 2013, Cortez-Dias and colleagues determined TC, LDL-C, HDL-C, and TG percentiles for a specific Portuguese population, primary health care users, but this has limited application due to sample bias (Cortez-Dias et al., 2013).

Biomarker percentiles are of extreme importance for the definition of reference intervals, being useful for giving the relative standing of an individual in a population. They are essentially the rank position of an individual. The percentiles calculation has the advantage that these are not strongly influenced by extreme values of the distribution (as the mean value), and do not requires normally distributed data, which means that can be calculated, even if the data are skewed (Altman, 1991).

Percentiles can be obtained by different strategies, including bootstrap methods that are increasingly being used in the medical literature, especially for non-Gaussian population's distributions or in the absence of any knowledge of a distribution. In a bootstrap, a set of data is randomly resampled with replacement, multiple times, and statistical conclusions are drawn from the data collection. The nonparametric bootstrap is a very computer-intensive method, but with a valuable application in the determination of confidence intervals of a quantile (e.g. 0.05 to 0.95) or percentile (e.g. 5^{th} to 95^{th}) (Henderson, 2005; Desharnais et al., 2015).

4. DYSLIPIDAEMIA AS CARDIOVASCULAR RISK FACTOR

Deaths from atherosclerotic CVD have declined in the past 30 years, but CVD is the leading cause of death among non-communicable diseases and disabilities worldwide, both in developed and developing countries, accounting for 47% of deaths in Europe (Nichols et al., 2012; GBD 2013 Mortality and Causes of Death Collaborators et al., 2015). CVD has a multifactorial aetiology with a number of potentially modifiable risk factors. Dyslipidaemia, hypertension (HT) and cigarette smoking are three well-known major yet modifiable, risk factors for CVD. However, several studies have demonstrated that is possible to reduce CVD events through risk factor modification and prevention (Gielen and Landmesser, 2014).

Epidemiological studies have linked CVD to increasing values in serum lipids, such as TC, non-HDL-C, LDL-C and TG, and also to low concentration of HDL-C; alone and together, these changes contribute to the development of atherosclerosis (Perk et al., 2012; Catapano et al., 2016). On the other hand, the central role of dyslipidaemia as a major contributor to CVD risk has been highlighted by the global case-control INTERHEART study (Yusuf et al., 2004), in which lipoprotein profile represented by varying apolipoprotein apoB/apoA1 strata had the highest population-attributable risk (54%) and the highest odds ratio (OR) with each 1 standard deviation (SD) difference [1.59; 95% confidence interval (1.53-1.64)]. The apoB/apoA1 ratio has been suggested as superior to any of the cholesterol ratios for estimation of the risk of acute myocardial infarction (MI) in all ethnic groups, in both sexes, and at all ages (Yusuf et al., 2004).

The increasing incidences of obesity and type 2 diabetes mellitus might significantly contribute to the CVD mortality rate high rate (Kohli et al., 2014; Vaduganathan et al., 2015), carrying a high proportion of patients with complex lipid abnormalities, which are not restricted to elevated LDL-C or TC levels but often comprise reduced levels of HDL-C, and/or elevated TG, non-HDL-C and small dense LDL (sdLDL) (Sardinha et al., 2012).

There are three categories of disease prevention, namely primary, secondary, and tertiary prevention (Figure 4). Primary prevention refers to the modification of risk factors associated with disease development, secondary prevention refers to the disease progression control, and tertiary prevention refers to the reduction of the consequences of disease regarding the functional status and quality of life (Kones, 2011; Vaduganathan et al., 2015). Dyslipidaemia, HT, diabetes and metabolic syndrome are clearly included in the primary prevention. Note that these preventions require

population-based strategies and collaboration through a diverse health system.

There is a great evidence that LDL-C lowering therapies (primarily statins) substantially reduce risk of CVD events in patients at high risk of any type of major vascular event: for every 1 mmol/L (39 mg/dL) decrease in LDL-C, the risk of major cardiovascular events is decreased by 21% (Bonovas et al., 2011). Guidelines are using CVD risk to guide treatment strategies, due to evidences that the atherosclerotic process began in infancy (or even earlier), with genetic factors contributing to the CHD development (Goff et al., 2014; Catapano et al., 2016). For instance, the Cardiovascular Risk in Young Finns followed-up study (Juonala et al., 2010) demonstrated that physical inactivity and reduced fruit intake are correlated with accelerated carotid intima thickness. They showed that the correction of these risk factors during childhood attenuated the risk for progression during adulthood. So, these results evidenced the earlier the prevention is started, the lower the risk of atherosclerotic disease. Recent studies refers to the prevention of risk factors related to means health behaviours, such as infant health, smoking, physical activity, body weight, environmental pollution, and diet, as a primordial prevention in the basis of the CVD prevention (Figure 4) (Vaduganathan et al., 2015; Hong et al., 2017).

As dyslipidaemia is one of the major CVD risk factors, knowing the lipid profile of a population will help to predict cardiovascular mortality trends for the following years and to design preventive strategies to cope with this health problem. The prevalence of dyslipidaemia can vary across population groups according to nationality, ethnicity, genetics and socio-cultural and economic factors. Because the population is aging and health behaviours changing, a periodic assessment of the prevalence of cardiovascular risk factors is necessary. Recently, Hong and colleagues (Hong et al., 2017) highlighted how challenging is the assessment of risk due to limitations of contemporary data. With regard to the Portuguese reality, the last evaluation was in 2013, where Cortez-Dias and colleagues characterised the dyslipidaemia patterns in their sample but this does not correspond to the general population (Cortez-Dias et al., 2013). So the last assessment of the dyslipidaemia patterns in Portugal was performed in 2001 in a project funded by Becel (Instituto de Alimentação Becel, 2002). No other population study has been done for dyslipidaemia since that time.

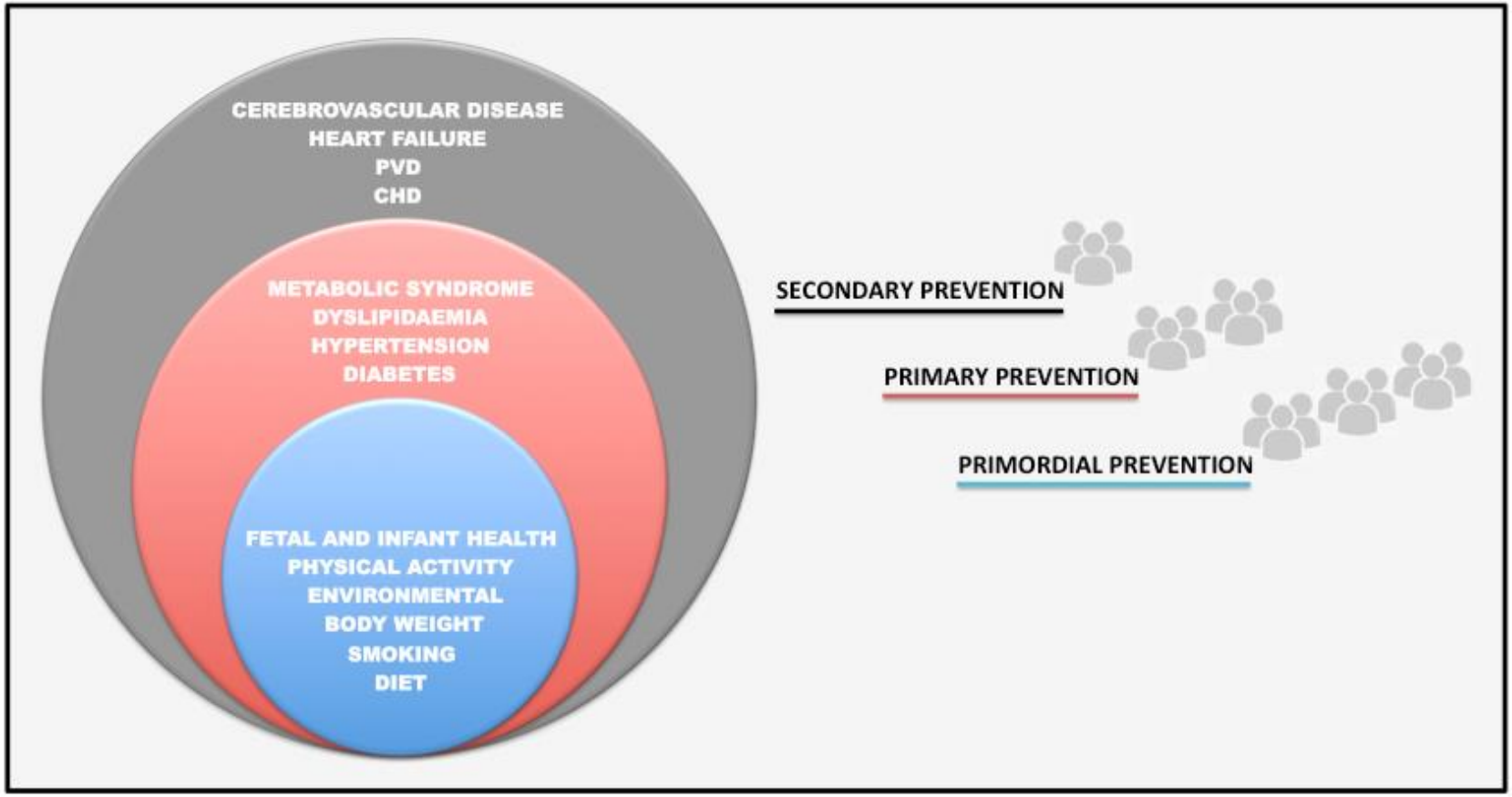

Figure 4 – Cardiovascular disease prevention categories. Primary prevention (pink) refers to the modification of risk factors associated with disease development, and secondary and tertiary preventions (grey) refers to the disease progression control and the reduction of the consequences of disease regarding the functional status and quality of life, respectively. Primordial prevention (blue) makes up the base and refers to the prevention of risk factors for disease from developing, which means health behaviours. Adapted from Vaduganathan et al., 2015. PVD, peripheral vascular disease; CHD, coronary heart disease.

4.1. Monogenic dyslipidaemias

Of many independent cardiovascular risk factors that have been identified, namely, dyslipidaemia, HT, diabetes, sedentary life style, overweight/obesity, inadequate diet and smoking, all have a common link: all could be modifiable and their correct management could contribute to the CVD prevention. In contrast, other CVD risk factors are considered non-modifiable, like genetic risk factors. However, the genetic associated risk can be prevented if early identified, making genetic studies a priority in cardiovascular genetics research. In fact, numerous genetic variants from rare to common with significant effects in plasma lipid and lipoprotein levels have been identified trough recent technological advances (e.g. genome-wide association studies (GWAS) and next-generation sequencing (NGS)) (Hegele et al., 2015; Dron and Hegele, 2016).

Monogenic dyslipidaemias are caused by changes in a single gene (Kingston, 1989) associated with the lipid metabolism, and are sufficient for the disease to be expressed. They are classified according to the primary lipid or lipoprotein disturbance, namely high or low levels of LDL-C, high or low levels of HDL-C, or high levels of TG (Hegele, 2009). The molecular basis of most of them is completely understood, most following Mendelian segregation patterns (Kingston, 1989; Rahalkar and Hegele, 2008; Hegele, 2009; Fu et al., 2013; Farhan and Hegele, 2014). In 2016, Dron and Hegele (Dron and Hegele, 2016) mentioned 27 monogenic dyslipidaemias already identified in a total of 25 genes (Table 2), referring that despite all are defined by extreme lipid or lipoprotein values, individuals could present discrete signs and symptoms. Although, most dyslipidaemia diagnosis and treatment can be made based on clinical and biochemical profiles, in some cases, mutations in different genes can cause very similar phenotype (Medeiros et al., 2016; Chora et al., 2017b), or different phenotypes can result from different mutations in the same gene (Cohen et al., 2005, 2006; Zhang et al., 2007; Di Leo et al., 2008), revealing the importance of a complete molecular characterisation. There are evidences that CVD risk has a 22-fold increase in patients with detected causative mutation for hypercholesterolaemia compared to individuals with the same LDL-C values but no mutation. Ference and colleagues (Ference and Mahajan, 2013) showed that lowering LDL-C levels from earlier in life can prevent or delay the development of atherosclerosis, improving the clinical benefit of therapies that lower LDL-C levels. Still, very recently, Amor-Salamanca and colleagues (Amor Salamanca et al., 2017) stated that FH clinical criteria (either Simon Broome nor Dutch Lipid Clinic Network) do not accurately classify FH patients with acute coronary syndrome, recommending the molecular study, especially in young patients with acute coronary syndrome and high LDL-C levels. Considering that nowadays high-throughput DNA

sequencing technologies are available with decreasing costs over time, a more accurate molecular diagnosis is already facilitated.

The study of all known dyslipidaemia-associated genes could explain the origin of lipids and lipoproteins variations within a population, while the study of individuals without a known cause for their dyslipidaemia can lead to the identification of a novel gene and a novel therapeutic target.

Table 2 – Monogenic dyslipidaemia-causing genes (Johansen et al., 2014).

Phenotype	Gene	Lipid disorder
High LDL-C	***LDLR***	Autosomal dominant FH
	APOB	Autosomal dominant FH
	PCSK9	Autosomal dominant FH
	STAP1	Autosomal dominant FH
	APOE[a]	Autosomal dominant FH phenocopy
	LDLRAP1	Autosomal recessive FH
	LIPA	Cholesterol ester storage disease and Wolman syndrome
	ABSG5/8	Sitosterolaemia
Low LDL-C	***MTTP***	Abetalipoproteinaemia
	APOB	Homozygous hypobetalipoproteinaemia
	PCSK9	Hypobetalipoproteinaemia, PCSK9 deficiency
	SAR1B	Anderson disease, chylomicron retention disease
	ANGPTL3	Familial combined hypolipidaemia
High HDL-C	***CETP***	Hyperalphalipoproteinaemia
	LIPC	Hepatic lipase deficiency
	SCARB1	SCARB1 deficiency
Low HDL-C	***ABCA1***	Tangier disease, primary familial hypoalphalipoproteinaemia
	APOA1	Primary hypoalphalipoproteinaemia
	LCAT	LCAT deficiency
High Triglycerides	***LPL***	Chylomicronaemia, LPL deficiency, severe hypertriglyceridemia
	APOC2	Chylomicronaemia, apoC2 deficiency, severe hypertriglyceridaemia
	APOA5	Severe hypertriglyceridaemia, apoA5 deficiency
	LMF1	Severe hypertriglyceridaemia, LMF1 deficiency,
	GPIHBP1	Severe hypertriglyceridaemia, hyperlipoproteinaemia type 5
	GPD1	Infantile hypertriglyceridaemia
Low Triglycerides	***APOC3***	ApoC3 deficiency

LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FH, Familial hypercholesterolaemia; PCSK9, proprotein convertase subtilisin kexin 9; SCARB1, scavenger receptor B1; LCAT, Lecithin:cholesterol acyltransferase; LPL, lipoprotein lipase; apoC2, apolipoprotein C2; apoA5, apolipoprotein A5; LMF1, lipase maturation factor 1; apoC3, apolipoprotein C3 .

[a]*APOE* gene is also associated to the dysbetalipoproteinaemia.

4.1.1. Familial Hypercholesterolaemia

FH is one of the most common diseases of the lipid metabolism, with an autosomal dominant inheritance pattern. The FH was the first genetic disease of the lipid metabolism to be molecularly characterised (Goldstein et al., 1985). FH patients usually have high concentrations of plasma LDL-C (above the 95th percentile for gender and age), leading to a premature CVD (e.g. MI) (Goldstein et al., 1995).

In 1972, Brown and Goldstein initiated their study with homozygous FH (HoFH) postulating that it would be presented as an autosomal dominant disorder and speculating that would be a defect in a protein involved in the final process of the cholesterol synthesis. A genetic defect in the feedback mechanism of the regulation of cholesterol synthesis was never described before, so it was a great challenge to prove this theory (Goldstein et al., 1985). They performed the molecular characterisation of the first mutation in the LDL receptor gene (*LDLR*) and they were awarded the Nobel Prize for medicine in 1985 with this discovery (Brown and Goldstein, 1986).

Nowadays, it is known that genetic causes for FH are loss-of-function mutations in the *LDLR* (Stenson et al., 2014) or *APOB* (Innerarity et al., 1990; Motazacker et al., 2012; Alves et al., 2014) genes, and gain-of-function mutations in the *PCSK9* gene (Abifadel et al., 2003) (Figure 5). However, FH is mainly due to loss of function mutations in the *LDLR* or *APOB* genes. Gain-of-function mutations in the *PCSK9* gene are a rare cause of FH. Indeed, a very rare autosomal recessive hypercholesterolaemia is caused by mutations in the low-density lipoprotein receptor adaptor protein 1 (*LDLRAP1*) gene, encoding for a cytosolic protein that interacts with the cytoplasmic tail of the LDLR (Figure 6). There are mutations in two other genes related to the lipid metabolism causing FH phenotype, namely Cholesterol 7 alpha-hydroxylase (*CYP7A1*) (Soutar and Naoumova, 2007) and Sterol regulatory element-binding protein 2 (*SREBP-2*) genes (Miserez et al., 2002). The CYP7A1 protein is involved in the first step of hepatic cholesterol metabolism and bile acid synthesis, while the SREBP-2 protein plays a role in the regulation of the metabolism of fatty acids and cholesterol. However, the evidences are neither strong nor conclusive (Soutar and Naoumova, 2007). Additionally, as several studies have been performed to identify new genes causing FH, signal transducing adaptor protein family 1 (*STAP1*) gene (Fouchier et al., 2014) and patatin-like phospholipase-domain-containing family (*PNPLA5*) gene (Lange et al., 2014) have yet to be confirmed as independently FH-causing genes. The function of STAP1 protein is largely unknown, but it has been suggested that functions as a phosphoinositide-binding domain and facilitates the association of STAP1 with membranes (Fouchier et al., 2014). On the other hand, the PNPLA5 protein is known to be involved in the

TG hydrolysis (Kurat et al., 2006).

FH is the most common inherited lipid disorder associated with premature CHD (pCHD), with a frequency around 1:400/500 in most populations (Nordestgaard et al., 2013). However, recent population surveys reported that heterozygous FH (HeFH) has a frequency of 1:217 (Benn et al., 2016), suggesting an underestimated prevalence. Differences in molecular diagnostic methodologies, and also in the clinical criteria applied might also contribute to the discrepancies in the prevalence of mutation-causing disease across different cohorts (Benn et al., 2012; Futema et al., 2012; Motazacker et al., 2012; Bertolini et al., 2013; Nordestgaard et al., 2013).

There are two mainly used criteria for clinical diagnosis of FH, one from the Dutch Lipid Clinic Network (DLCN) (Austin et al., 2004), and another one from National Institute for Clinical Health and Excellence (NICE)-endorsed Simon Broome (SB) Register (Simon Broome Register Group, 1991), which is the one used in the Portuguese FH Study. The Simon Broome criteria take into consideration cholesterol concentrations, clinical characteristics, molecular diagnosis, and family history of hypercholesterolaemia and/or pCHD. A “possible” diagnosis of FH is considered when individuals have TC above 290 mg/dL (260mg/dL for children) or LDL-C above 190 mg/dL (155 mg/dL for children) and family history of pCHD or hypercholesterolaemia, while the “definite” diagnosis of FH is made if a patient has elevated cholesterol levels and tendon xanthomata, or a mutation already identified. The DLCN is a modification of the SB criteria using a point system (DLCNS score) based on cholesterol levels, personal and family history of pCHD, physical examination and detected mutations. Total point scores of greater than 8 is considered "definite" diagnosis of FH, 6-8 is "probable" diagnosis of FH, and 3-5 is "possible" diagnosis of FH.

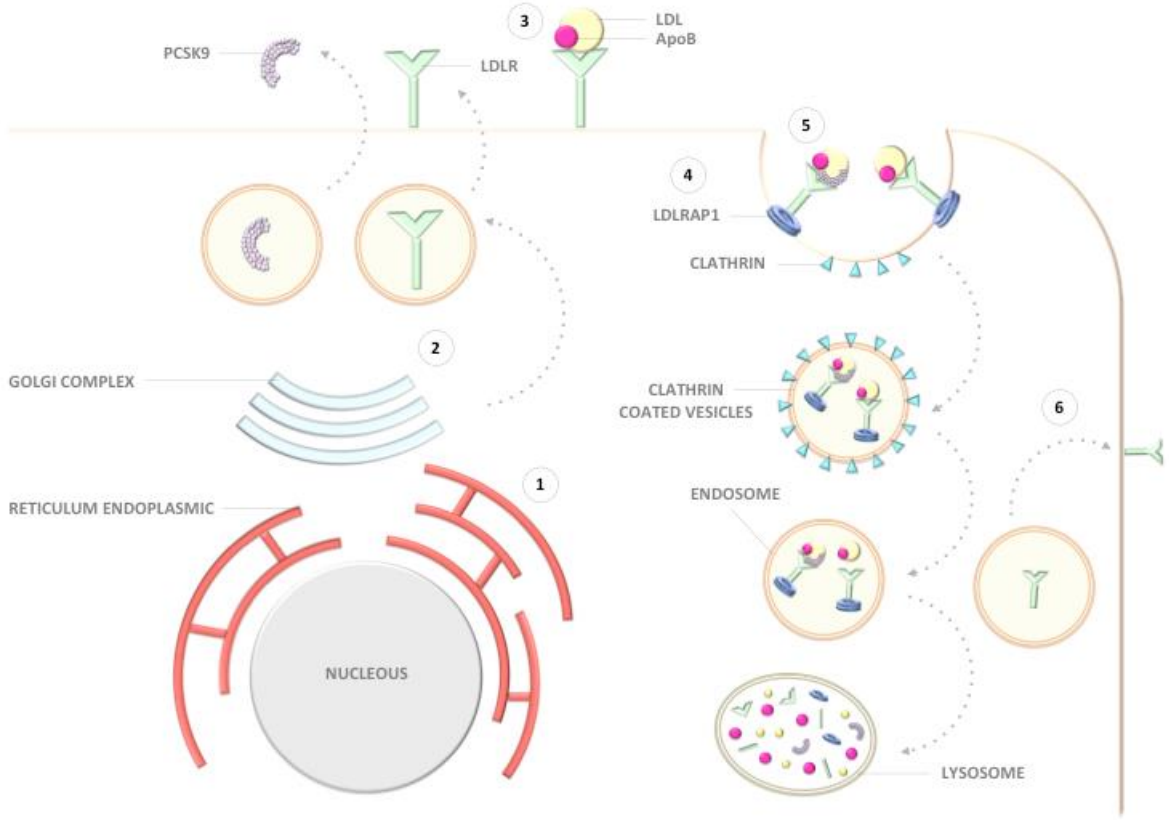

Figure 5 – Low-density lipoprotein receptor (LDLR) pathway and FH causing mutations. The low-density lipoprotein (LDL) particles are removed from plasma via LDLR through apolipoprotein B (apoB100) on the surface of LDL. The LDLR internalises the complex LDL particle-LDLR into a clathrin vesicles. These clathrin vesicles are further fused to form endosomes. After endocytosis, occurs the disassociation of LDL from the LDLR by acidic conditions of the endosome (by lowering the pH, a dissociation of the complex within the endosome occurs, leading to the LDLR recycling back to the cell membrane. LDL particles migrate to a lysosome, where apoB is degraded to amino acids and the cholesteryl esters hydrolysed by lysosomal acid lipase (LIPA) into free cholesterol, to be further incorporated into cell membranes, or used into bile acids, or synthesis of steroids (Liscum and Underwood, 1995; Rifai et al., 1999)

The protein propotein convertase subtilisin/kexin type 9 (PCSK9) protein is involved in regulation of the degradation of the LDLR protein, preventing it from being recycled to the cell surface. Known mechanisms causing familial hypercholesterolemia due to LDLR are numbered from**1** to **6**. **1** and **2**) a failed LDLR protein synthesis or a failed LDLR protein transport to cell membrane surface by precursor proteins can occurs (**3**) a defective binding domain of the LDLR or a familial defective apoB can impairs the ability of the apoB to bind with the LDLR (**4**) the LDLR adaptor protein (LDLRAP) can impairs the ability of the LDLR to interact with LDL particles to extract cholesterol (**5**) gain-of-function mutations in the *PCSK9* can inhibit LDLR function and increase the degradation of LDLR (**6**) a failed surface presentation can also occurs. Adapted from Alves, 2014.

4.1.1.1. Portuguese Familial Hypercholesterolaemia Study

The Portuguese FH Study has been successfully implemented since started in 1999. The major identification and molecular characterisation of patients with FH in Portugal started with this study (Bourbon et al., 2006). Only a few identified cases already existed before. The main goals of the Portuguese FH Study are the estimation of the prevalence and distribution of FH in Portugal, as well as the clinical and molecular characterisation of the FH patients, and also the characterisation of their CVD pathophysiology (Bourbon et al., 2006).

As mentioned previously, the molecular diagnosis of FH allows the correct identification of the cause of dyslipidaemia, important for prognosis, family counselling, and treatment decision (e.g. a more aggressive lipid-lowering therapy), with a positive impact on the CVD risk in the affected individuals (Bourbon et al., 2006, 2008).

The Portuguese FH Study includes a biochemical and a molecular study and is divided into five phases (Medeiros et al., 2010, 2016). Phase one includes DNA extraction, screening for the most common *APOB* mutations (fragments of exon 26 and exon 29), and the study of the promoter, splicing and coding regions of the *LDLR* gene. Phase two comprises the study of large rearrangements by multiplex ligation-dependent probe amplification (MLPA) method. Phase three is the study of five exons (exons 1, 2, 4, 7, and 9) and flanking regions of *PCSK9* (regions where pathogenic variants have been described). In more severe cases, the whole *PCSK9* gene is investigated. Phase four includes the study of promoter, all exons, as well as flanking regions of the *APOB* gene (Alves et al., 2014). In the last phase, phase five, functional *in vitro* studies are performed for missense mutations, in frame deletions/insertions, and splicing variants without previous functional studies (Benito-Vicente et al., 2015). The phases one and two are always performed for all patients, while phases three and four are only included when no putative mutations is detected in the previous phases. When applicable, other dyslipidaemia-causing genes are investigated. In this sense, a basic panel for targeted sequencing with the three proven FH-causing genes, *LDLR*, *APOB*, and *PCSK9*, and the *APOE* and *LDLRAP1* genes, as well as the 6 LDL-C score SNPs of polygenic dyslipidaemia, is almost implemented in the Portuguese FH Study, being the inclusion of other genes planned for the near future. All novel variants found are classified as pathogenic, likely pathogenic, benign, likely benign, or variant of unknown significance (VUS), according to Chora et al. (Chora et al., 2017a).

The molecular study performed in the Portuguese FH Study until 2015 has identified 668 individuals with monogenic cause for their dyslipidaemia, who are currently receiving

family counselling and treatment accordingly. During that period, some patients with milder phenotype than that indicated by the SB criteria were also included in the EPHF. Despite all effort of the Portuguese FH Study, the genetic cause in some individuals with clinical diagnosis of FH remains unexplained.

4.1.2. Other monogenic dyslipidaemias

To date, high-throughput DNA sequencing in families with previously uncharacterised monogenic dyslipidaemias have failed to reveal new genes for regulation of plasma lipids, suggesting that a diagnosis should be focuses primarily on genes already known to be involved in the lipid and lipoprotein metabolism. Studies using NGS as a novel approach have shown that a few patients with clinical diagnosis of FH were carriers of pathogenic or likely pathogenic variants in the *APOE* (Marduel et al., 2013), *LIPA* (Stitziel et al., 2013) and *ABCG5 and 8* (Rios et al., 2010) genes, all acting within lipoprotein metabolic pathways. These are dyslipidaemia-causing genes for dysbetalipoproteinaemia, lysosomal acid lipase deficiency (LALD) and sitosterolaemia, respectively, where affected individuals might present clinical phenotype suggestive of FH. In fact, it has been suggested the inclusion of these genes on the screening of FH whenever possible (Hegele et al., 2015; Chora et al., 2017).

On the other hand, alterations in some of FH-causing genes are also related to extreme reduction levels of LDL-C. For instance, loss-of-function mutations in *PCSK9* (Cohen et al., 2005) and *APOB* genes (Di Leo et al., 2008) have been reported in individuals with hypocholesterolaemia, and although loss-of-function mutations in *PCSK9*, in heterozygous or homozygous state, protect against coronary heart disease (Cohen et al., 2006; Zhang et al., 2007), loss-of-function mutations in *APOB* homozygote state lead to a serious condition called hypobetalipoproteinemia, where patients can have neuronal dysfunctions. Recently, other genes have been also implicated in a hypocholesterolaemia phenotype as angiopoietin-like 3 (*ANGPTL3*) gene (Calandra et al., 2011) and secretion associated, Ras related GTPase 1B (*SAR1B*) gene (Burnett and Hooper, 2008; Tarugi and Averna, 2011). ANGPTL3 protein is predominantly expressed in the liver, where play a role in the trafficking of energy substrates to either storage or oxidative tissues in response to food intake, and by suppressing plasma TG clearance via inhibition of LPL activity (Robciuc et al., 2013; Tikka et al., 2014). SAR1B protein is present in the enterocytes, where aids in the transport of chylomicrons (Shoulders et al., 2004). Both genes are now under study for the development of therapeutic approaches. Beyond these, homozygous or compound heterozygous mutations in the *MTTP* gene encoding for the microsomal triglyceride transfer

protein (MTP), a protein that plays a key role on the assembly and secretion of lipoproteins containing apoB in both liver and intestine, cause abetalipoproteinaemia, an autosomal recessive disorder characterised by a virtual absence of apoB-containing lipoproteins (Lee and Hegele, 2014).

Besides an elevation of LDL-C levels, low HDL-C is considered an independent risk factor for CVD (Catapano et al., 2016). In fact, functional alterations that impair HDL production or enhance its catabolism have been studied, revealing that there are rare alleles with major phenotypic effects that contribute significantly to low HDL-C in the general population. Variants causing rare genetics forms of HDL deficiency were identified in genes directly or indirectly involved in the lipid metabolism, namely *APOA1* (Cohen et al., 2004), *LCAT* (Cohen et al., 2004), and the adenosine triphosphate binding cassete transporter A1 (*ABCA1*) (Hong et al., 2002; Cohen et al., 2004; Frikke-Schmidt et al., 2008) genes. *ABCA1* gene code for a ABCA1 protein that is predominantly high in hepatocytes and macrophages cells, where plays an important role in the HDL-C metabolism, by moving cholesterol and phospholipids across the cell membrane to be further picked up by the apoA1 (Oram, 2003; Brunham et al., 2006; Tall et al., 2008), the major apolipoprotein of the HDL particle. Patients with *ABCA1* mutation have HDL-C deficiency (in heterozygosity) or Tangier disease (in homozygosity), being Tangier a serious disorder leading to premature CVD (Fredrickson, 1964). Conversely, mutations in the *CETP* (Inazu et al., 1990), *LIPC* (Connelly and Hegele, 1998; Tilly-Kiesi et al., 2004), and Scavenger Receptor Class B Member 1 (*SCARB1*) (Zanoni et al., 2016) genes were associated to extreme high levels of HDL-C. SCARB1 protein is a plasma membrane receptor for HDL particle, mediating cholesterol transfer to and from HDL (Ji et al., 1999). Despite the cardioprotective function of HDL be largely attributed to its ability to facilitate the transport of cholesterol from peripheral tissues to the liver, as mentioned previously recent data suggest that the relationship between high levels of HDL-C and cardiprotective effect it does not necessarily true for very high HDL-C levels, particularly when a large HDL subfraction particles are predominantly.

Another condition also associated with increased CVD risk (and not less important) is the hypertriglyceridaemia. High levels of TG may arise as a result of defective metabolism of TG-rich lipoproteins and their remnants, as consequence of rare variants with large effects in genes directly or indirectly involved in its pathway (Nordestgaard and Varbo, 2014). As mentioned before, several studies have been stated a causal association between raised TG and CVD (Hokanson and Austin, 1996; Sarwar et al., 2007). Rare autosomal recessive monogenic disorders that causes extreme levels of TG (Johansen and Hegele, 2011, 2012; Johansen et al., 2011; Basel-Vanagaite et al., 2012;

Surendran et al., 2012; Lewis et al., 2015) can be found in the *LPL*, *APOC2/3*, *APOA5*, lipase maturation factor 1 (*LMF1*), glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 (*GPIHBP1*), and glycerol-3-phosphate dehydrogenase-1 (*GPD1*) genes.

The protein encoded by *LMF1* gene is involved in the maturation and transport of the LPL through the secretory pathway (Péterfy et al., 2007), while GPIHBP1 protein plays a major role in transporting LPL trough endothelial cells (Beigneux et al., 2007), and the GPD1 in the TG synthesis (Basel-Vanagaite et al., 2012). The most common disorder is the Familial chylomicronaemia, where the majority are due to LPL deficiency (Rahalkar et al., 2009). Importantly, severe hypertriglyceridaemia due to monogenic chylomicronaemia can also cause recurrent pancreatitis, with several serious complications, which can even be fatal (Sandhu et al., 2011). If on the one hand, homozygous or compound heterozygous for pathogenic or likely pathogenic variants in the *LPL* gene have been reported to cause LPL deficiency, mutations in the *APOC2*/*3*, *APOA5*, *LMF1*, *GPIHBP1* or *GPD1* genes can also be implicated in the chylomicronaemia phenotype (Hegele et al., 2014). This is not surprisingly, taking into account that all proteins encoded by those genes are somehow related to the LPL enzyme, a key enzyme involved in the hydrolysis and removal of TG from plasma. There are also mutations associated to low TG and reduced risks of ischemic vascular disease and ischemic heart disease. Jorgensen and colleagues (Jørgensen et al., 2014) found loss-of-function mutations in *APOC3* gene in individuals with reduced levels of nonfasting TG, with evidences that the cumulative incidences of CHD were reduced in heterozygotes as compared with noncarriers of *APOC3* mutations.

4.2. Polygenic hypercholesterolaemia

In the last years, GWAS studies have identified several loci significantly associated to the lipid metabolism. Between 2010 and 2013 a meta-analysis of GWAS studies data from the Global Lipid Genetic Consortium (GLGC) identified 157 common variants influencing plasma lipid concentrations, and determined the small effect of each polymorphism for each trait (TC, LDL-C, HDL-C, and TG) (Teslovich et al., 2010; Willer et al., 2013). From this evidence, in 2013 Talmud and colleagues (Talmud et al., 2013) suggested that the small-effect LDL-C raising alleles might have a cumulative effect, leading to an increase in LDL-C, even at the level of FH range. Thus, they hypothesised if some of clinically diagnosed cases of FH could possibly have a polygenic cause. To prove this, they used a 12-single nucleotide polymorphisms (SNP) LDL-C genetic risk score (GRS), by the sum of the effect sizes (weight) of the raising alleles, where the effect size was the beta

coefficients from statistical models previously determined in the GLGC study. This LDL-C GRS was based on polymorphisms in the solute carrier family 22 member 1 (*SLC22A1*), human hemochromatosis protein (*HFE*), myosin regulatory light chain interacting protein (*MYLIP*), NYN domain and retroviral integrase containing (*NYNRYN*), ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 4 (*ST3GAL4*), cadherin EGF LAG seven-pass G-type receptor 2 (*CELSR2*), *APOB*, *ABCG5/8*, *LDLR*, *PCSK9* and *APOE* genes. By modelling, they obtained 95% of probability of the individuals from the UK general population (Whitehall II (WHII) Study) that was above the 25th percentile for the LDL-C GRS, have a polygenic cause for their increased LDL-C, thus supporting their findings. They further compared the weighted LDL-C GRS frequency distribution among general population and UK FH/M+ patients and FH/M- patients, and found that the LDL-C GRS was statistically significantly higher in FH/M- patients. They validated this 12-SNP LDL-C GRS by demonstrating that were statistically significantly differences between FH cohorts and the WHII controls, and by replicating analysis using a FH cohort from a different population (Belgium). They also showed that FH/M- patients had statistically significantly higher score than FH/M+ patients. Although different, the LDL-C GRS in FH/M+ patients was still higher than controls, suggesting that even in patients with a disease causing mutation, this polygenic contribution could also be found, reflecting in a variable FH phenotype.

In 2015, the same research group investigated the possibility of additional SNPs improve the discrimination, but results showed that increasing the number of SNPs to 33 did not improve the ability of the LDL-C GRS to discriminate between FH/M- patients and controls (Futema et al., 2015). More than that, they also analysed the influence of fewer SNPs, and showed that using 6 SNPs (*CELSR2/SORT1* (rs629301), *APOB* (rs1367117), *ABCG5/8* (rs4299376), *LDLR* (rs6511720) and *APOE* (rs7412 and rs429358)) from the previous 12-SNP LDL-C GRS performed optimum discrimination as well. In this study, they included six cohorts from different countries, including a paediatric cohort, demonstrating that a 6-SNP LDL-C GRS was consistently distinguish FH/M- patients from general population controls. Overall, values for the 6-SNP LDL-C GRS (mmol/L) were 0.708 for FH/M- patients, 0.656 for FH/M+ patients, and 0.632 for the WHII controls. From FH/M- patients above the first quartile (25th percentile) (88%), 36% had a score above the top quartile (75th percentile) of the WHII LDL-C GRS distribution. Children FH/M- (Dutch cohort) presented the higher score (0.782), but results should be confirmed using a child population as controls.

Different genetic scores have been suggested to estimate the polygenic contribution by a fraction of millimole per litter, although using a different combination of single

nucleotide polymorphisms, even with changes to the originally proposed (Hegele et al., 2015; Paquette et al., 2017) Hegele et al. (Hegele et al., 2015) even suggested a genetic score for other lipid traits, namely HDL-C and TG. The applicability of the LDL-C genetic risk scores has been demonstrated by other populations and/or studies, (Kwon et al., 2015; Sharifi et al., 2016; Lamiquiz-Moneo et al., 2017), but contradictory results have been found (Sjouke et al., 2016; Minicocci et al., 2017). Nevertheless, none of these studies have validated the scores using a population-specific as a control.

A mutation in one of the three FH-causing genes is expected be found in approximately 30%-50% (Medeiros et al., 2010, 2016; Taylor et al., 2010) of patients with clinical diagnosis of possible FH. In those where a causative mutation cannot be found, a small proportion could have an unidentified monogenic dyslipidaemia with a large effect on the LDL-C, or a mutation in a novel FH-causing gene may be present. In the remaining, they must likely have a polygenic cause, with a greater than average number of common LDL-C raising variants with modest effect. A pure environmental cause is also possible specially with the degradation of life styles habits

5. GLOBAL AIMS OF THE THESIS

The CVD has a complex aetiology and pathology, resulting from a combination of potentially modifiable risk factors (cigarette smoking, sedentarism, overweight/obesity, inadequate diet, alcohol intake, HT, and dyslipidaemia), with non-modifiable risk factors (as gender, age and genetics). Several epidemiological studies have linked CVD to plasma/serum lipids and lipoproteins concentrations, reporting dyslipidaemia as a well-known major risk factor for the atherosclerosis development. Some of dyslipidaemias have genetic causes (monogenic dyslipidaemias) with an elevated CVD risk per se, like FH, while mostly mild to severe dyslipidaemias results from multiple genes with small effect (polygenic dyslipidaemias) that are more easily modulated by modification of the life habits. Control of major cardiovascular risk factors has been revealed to definitively decrease the risk of CVD, so the evaluation and characterisation of the population lipid profile is essential to know the dimension of the problem. Since CVD risk has been shown higher in FH patients with detected causative mutation, compared to patients without a genetic cause, it is reasonable to think in distinguish between monogenic dyslipidaemia and polygenic or environmental hypercholesterolaemia, which could contribute for a better patient managements (the best therapeutically measure for the best patient prognosis).

Taking all these aspects in consideration, it is clear the importance of a regular dyslipidaemia assessment in a population as well as the correct identification of the aetiology of the dyslipidaemia in order to implement specific interventions for CVD prevention. Specially, if taking into consideration that some causes are not completely explained, and that lipid levels may be result of various genetic alterations that could interact with non-genetic factors and modulate the phenotype at some level, the different causes should be sought and understood. In this context, the main goal of the present work was the biochemical and molecular characterisation of the dyslipidaemia in the Portuguese population.

As a first step it was planned to define the reference intervals and establish reference values for lipid metabolism biomarkers, so the lipid and lipoprotein gender and age-specific percentiles were estimated for the general Portuguese population using a rigorous methodology. Therefore, these estimated percentiles values were compared with the percentiles from different populations, by using a very visual and feasible method for comparison analysis.

In a second step, it was assumed the reference values based on the percentiles previously determined, to characterise the lipid profile and the distribution of lipids and lipoproteins in the Portuguese population. Here, was also analysed the association among lipids and non-lipid/other cardiovascular risk factors. The investigation of the possible monogenic causes for hypercholesterolaemia or hypocholesterolaemia were then programmed to identify and characterise the extreme dyslipidaemia phenotypes in a general Portuguese population. At this point, the molecular study of those individuals in the extreme percentiles for lipid metabolism biomarkers were performed, namely TC and/or LDL-C, HDL-C, TG, and apoB.

Finally, and in an attempt to explore the causes for the FH phenotype, was thought to analyse the FH patients from the past 17 years of the Portuguese Familial Hypercholesterolaemia Study: 1) by assessing all the results of the Portuguese FH Study (EPHF) from 1999 to 2016, presenting all identified monogenic and polygenic causes for hypercholesterolaemia in the EPHF cohort, for both children and adults; 2) and by validating the LDL-C GRS in the general Portuguese population, using a 6-LDL-C-associated SNPs previously determined in a UK study, and exploring its applicability for the polygenic hypercholesterolaemia characterisation in the EPHF cohort, while at the same type results using different clinical FH criteria, the DLCN and SB criteria, were compared in a adult cohort.

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Chapter 2

ESTABLISHMENT OF LIPID METABOLISM REFERENCE VALUES BASED ON POPULATION SPECIFIC PERCENTILES

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ABSTRACT

Establishment of population specific, age and gender, reference intervals are recommended for a better interpretation of clinical laboratory tests and for patient care. The aima was to determine lipid and lipoprotein percentiles for the Portuguese population and to compare it with other population studies. Percentiles for lipid biomarkers were estimated using the e_COR study population, by bootstrapping, and compared with other population percentiles, by plotting the percentile graphs from each study together with the estimated percentiles and corresponding estimated 95% confidence intervals. A total of 866 individuals were included for analysis. The 5^{th}, 10^{th}, 25^{th}, 50^{th}, 75^{th}, 90^{th} and 95^{th} percentiles were obtained for total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), apolipoprotein A1 (apoA1), apolipoprotein B (apoB), small, dense LDL-C (sdLDL-C), lipoprotein(a) [Lp(a)], non-HDL-C, apoB/apoA1 and sdLDL/LDL ratios, and remnant cholesterol. Values for the 50^{th} percentile for TC and LDL-C are similar to the European Cardiology Society recommended values. The remainder differ or there are no reference values for evaluation. Comparison of percentiles between the e_COR Study and other populations showed relevant differences. We provided for the first time reference values for lipid biomarkers for the Portuguese population, based on lipid percentiles; the 25^{th}-75^{th} percentiles are in general within recommend values and above the 90^{th} or below 10^{th} (HDL-C, apoA1) are considered at risk values. The sdLDL-C percentiles are the first to be established for a population in Europe. We also showed a very visual and feasible method for comparison analysis of the percentile values. We strongly encourage the estimation of population-specific reference values, for the definition of optimal and at risk values.

KEYWORDS

Dyslipidaemia; Lipid biomarkers; Lipid percentiles; Reference values; at risk values.

1. INTRODUCTION

Cardiovascular disease has a multifactorial aetiology, but the majority of the risk factors are potentially modifiable, such as dyslipidaemia (Baigent et al., 2005; Gielen and Landmesser, 2014). The risk factor profile can vary across population groups according to nationality, ethnicity, genetics and socio-cultural and economic factors, so it is important to know the profile of each population. This information will help to predict cardiovascular mortality trends for the following years and to design preventive strategies to cope with this important health problem (Murray et al., 1997; Yusuf et al., 2001a, 2001b; Joshi et al., 2008). Equally important is to establish population specific, age and gender, reference intervals for a better interpretation of clinical laboratory tests and for patient care (Clinical Laboratory and Standards Institute, 2010).

Biomarker percentiles are of extreme importance for the definition of reference intervals, being useful for giving the relative position of an individual in a population. They are essentially the rank position of an individual. The percentiles calculation has the advantage that these are not strongly influenced by extreme values of the distribution (as the mean value), and do not require normally distributed data, which means that they can be calculated even if the data are skewed (Altman, 1991).

Percentiles can be obtained by different strategies, including bootstrap methods that are increasingly being used in the medical literature, especially for non-Gaussian population distribution or in the absence of any knowledge of a distribution. In a bootstrap, a set of data is randomly resampled with replacement, multiple times, and statistical conclusions are drawn from the data collection. The nonparametric bootstrap is a very computer-intensive method, but with a valuable application in the determination of confidence intervals (CI) of a quantile (e.g., 0.05 to 0.95) or percentile (e.g., 5^{th} to 95^{th}) (Henderson, 2005; Desharnais et al., 2015).

Here, we provide for the first time the percentiles for lipid metabolism biomarkers of the Portuguese population, namely total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), apolipoprotein B (apoB), apolipoprotein A1 (apoA1), small dense LDL-C (sdLDL-C), lipoprotein(a) [Lp(a)], and also for non-HDL-C, apoB/apoA1 and sdLDL-C/LDL-C ratios, and remnant cholesterol, for the Portuguese population. We then compared these percentiles with those from a Portuguese primary care study (Cortez-Dias et al., 2013) and with a Spanish (Gómez-Gerique et al., 1999) and American populations studies (Contois et al., 1996b; Bachorik et al., 1997).

2. MATERIALS AND METHODS

2.1. Study population

All samples and demographic and clinical data used in the present study were obtained from the e_COR Study, a pre-designed and developed cross-sectional epidemiological study performed by our research group with the major aim to determine the prevalence of cardiovascular risk factors in the Portuguese population, and with a secondary aim to determine biochemical reference values for the Portuguese population (Bourbon et al., 2018). The e_COR study included 1,688 individuals, 848 men and 840 women aged between 18 and 79 years, from the Norte, Centro, Lisboa, Alentejo, and Algarve regions of Portugal. The e_COR Study was approved by the National Data Collection Commission and National Institute of Health (INSA) Ethic Committee, and participants gave informed consent to each aspect of the study (Supplementary data).

2.2. Biochemical analysis

Biochemical methods and conditions are described in Supplementary data. Non-HDL-C values and remnant cholesterol (fasting state) were calculated as previously described (Catapano et al., 2016; Nordestgaard et al., 2016). Non-HDL-C was calculated as TC minus HDL-C, while remnant cholesterol was calculated as TC minus LDL-C minus HDL-C.

2.3. Lipid and lipoprotein percentiles determination

2.3.1. Exclusion criteria

Subjects with selected characteristics known to affect lipid metabolism, such as medical history of diabetes, hyperthyroidism and hypothyroidism, use of lipid-lowering therapy, and age above 60 years (since of the individuals with age above 60, 80% were medicated for dyslipidaemia) were excluded from this study. So a total of 866 adults (426 men and 440 women aged between 18 and 59) were included in the determination of 5^{th}, 10^{th}, 25^{th}, 50^{th}, 75^{th}, 90^{th}, and 95^{th} percentiles for each lipid biomarker: LDL-C, HDL-C, TC, TG, apoA1, apoB and non-HDL-C and remnant cholesterol. Of these, 857 (98.96%) individuals were included in the percentiles estimation of sdLDL-C and 835 (96.42%) in the percentiles estimation of Lp(a), due to lack of sample.

2.3.2. Statistical analysis

The e_COR sampling design allowed the powerful estimation of the national prevalence of cardiovascular risk factors and hence sample size was calculated with this purpose. As a consequence, the total sample was not representative of the Portuguese population regarding age and gender distribution and could not be used directly to estimate percentiles of the parameters of interest. To overcome this, the chosen approach consisted of resampling, from the total sample, a high number of subsamples following a sampling scheme that respected both age and gender distribution of the Portuguese population across the regions (source of age and gender distribution (NUTS II) from CENSUS 2011, *Instituto Nacional de Estatística* (INE) (Instituto Nacional de Estatistica, Censos 2011)). The percentiles of interest were then estimated for each subsample, allowing the collection of a large number of estimated values for each percentile. From each collection, the median was used as the estimate of the percentile and percentiles 2.5^{th} and 97.5^{th} were used as limits of the CI for the estimate.

Due to the referred sampling design, before proceeding to the quantile estimation, it was necessary to assess whether the global sample could be considered a representative sample of the adult Portuguese population for each biomarker (Supplementary Figure 1). For that purpose, deviations from homogeneity were tested (Supplementary Figure 2). Firstly, departure from homogeneity of the distribution of lipid biomarkers among regions was tested within each age group and gender using Kruskal-Wallis non-parametric statistical test. For the age groups with evidence of lack of homogeneity, a Kolmogorov-Smirnov test was applied between regions to assess lack of homogeneity among pairs of regions. Regions for which the homogeneity hypothesis was not rejected were grouped and analysed as one individual stratum. Considering for each region/group of homogeneous regions the respective stratum weights, percentiles were estimated by bootstrapping: data were randomly resampled 50,000 times and the number of distinct bootstrap samples determined according to the stratum weights. Thus, the percentiles were estimated to be representative of the adult Portuguese population.

2.4. Comparison of percentiles

Heat colour matrices were constructed for a more general overview and comparison of percentiles between studies. For a more detailed comparison, we plotted the estimated percentiles and their 95% CI for the Portuguese population (e_COR Study) together with the percentile values from the primary health care (PHC) users in Portugal (Cortez-Dias et al., 2013), the *Dieta y Riesgo de Enfermedad Cardiovascular en España* Study (DRECE) (Gómez-Gerique et al., 1999) from Spanish population, and the

Framingham Offspring Study and the National Health and Nutrition Examination Survey III Study (NHANES III) (Contois et al., 1996b) from American populations.

Analyses were performed using R (version 3.1.2) software (R: The R Project for Statistical Computing). Figures and graphs for percentiles comparison among populations were created with the ggplot2 package within R (ggplot2: Elegant Graphics).

3. RESULTS

3.1. Reference values for lipid metabolism biomarkers

Percentiles for lipids and lipoproteins, namely TC, LDL-C, HDL-C, TG, apoA1, apoB, sdLDL-C, Lp(a), and for non-HDL-c, apoB/apoA1 and sdLDL-C/LDL-C ratios, and remnant cholesterol, were calculated for both genders and age groups (18-29, 30-39, 40-49, 50-59, and 18-59), being presented in Table 1. The 50th percentile (P50th) values for the overall population (18-59) were as follows: for TC, 189 mg/dL; LDL-C, 116 mg/dL; HDL-C, 54 mg/dL; TG, 83 mg/dL; apoA1, 147 mg/dL; apoB, 88 mg/dL; sdLDL-C, 25 mg/dL; Lp(a), 11 mg/dL; non-HDL-c, 134 mg/dL; apoB/apoA1, 0.56 mg/dL; sdLDL-C/LDL-C, 0.22 mg/dL and remnant cholesterol, 16 mg/dL. The 90th percentile (P90th) values were as follows: for TC, 232 mg/dL; LDL-C, 156 mg/dL; TG, 164 mg/dL; apoB, 118 mg/dL; sdLDL-C, 44 mg/dL; Lp(a), 53 mg/ dL; non-HDL-c, 181 mg/dL; apoB/apoA1, 0.92 mg/dL; sdLDL-C/LDL-C, 0.31 mg/dL and remnant cholesterol, 29 mg/dL;. For HDL-C and apoA1, the 10th percentile (P10th) values were 34 mg/dL and 116 mg/dL for men, and 45 mg/dL and 130 mg/dL for women, respectively.

Table 1 – Lipid and lipoprotein percentiles by gender and age group: 5th, 10th, 25th, 50th, 75th, 90th and 95th percentiles estimated for total cholesterol, LDL-C, HDL-C, TG, apoB, apoA1, sdLDL-C, Lp(a), non-HDL-C, apoB/apoA1, sdLDL-C/LDL C and remnant cholesterol.

Total cholesterol		**Percentile**						
Gender	**Age group**	**5th**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**
Men	18-29	123	129	146	166	184	209	221
	30-39	131	153	173	192	218	238	250
	40-49	160	163	190	213	232	253	260
	50-59	159	170	183	207	239	267	283
	18-59	**130**	**144**	**166**	**187**	**216**	**238**	**252**
Women	18-29	129	139	162	183	198	215	228
	30-39	147	156	172	181	204	233	244
	40-49	151	153	169	193	217	241	267
	50-59	160	165	179	207	220	244	254
	18-59	**145**	**155**	**171**	**189**	**212**	**229**	**243**
Total	**18-59**	**135**	**149**	**168**	**189**	**212**	**232**	**252**

LDL-C		**Percentile**						
Gender	**Age group**	**5th**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**
Men	18-29	59	67	82	102	121	140	146
	30-39	80	88	101	132	155	175	183
	40-49	92	101	117	143	158	169	180
	50-59	90	99	124	137	163	189	207
	18-59	**72**	**80**	**101**	**124**	**141**	**161**	**179**
Women	18-29	63	67	86	98	109	135	144
	30-39	74	82	95	109	126	141	154
	40-49	77	83	94	120	134	166	182
	50-59	79	86	110	126	154	172	180
	18-59	**68**	**78**	**90**	**102**	**123**	**145**	**175**
Total	**18-59**	**70**	**81**	**95**	**116**	**136**	**156**	**173**

HDL-C		**Percentile**						
Gender	**Age group**	**5th**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**
Men	18-29	33	39	43	50	57	65	72
	30-39	30	33	39	46	54	67	78
	40-49	30	34	39	47	55	62	73
	50-59	34	35	44	52	63	73	79
	18-59	**31**	**34**	**41**	**50**	**58**	**69**	**83**
Women	18-29	41	49	55	63	74	83	88
	30-39	43	46	53	62	74	80	84
	40-49	39	44	52	60	70	82	87
	50-59	39	41	46	58	64	79	84
	18-59	**41**	**45**	**53**	**61**	**73**	**81**	**87**
Total	**18-59**	**33**	**37**	**45**	**54**	**64**	**78**	**84**

Triglycerides		**Percentile**						
Gender	**Age group**	**5th**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**
Men	18-29	40	46	55	69	95	131	154
	30-39	47	55	71	97	140	187	230
	40-49	56	59	83	116	176	224	294
	50-59	52	57	66	106	126	174	224
	18-59	**46**	**52**	**64**	**89**	**129**	**182**	**230**
Women	18-29	39	44	61	77	101	139	156
	30-39	41	44	59	79	102	130	160
	40-49	43	46	54	79	106	144	174
	50-59	48	53	69	82	107	175	186
	18-59	**41**	**46**	**60**	**79**	**103**	**139**	**166**
Total	**18-59**	**43**	**48**	**62**	**83**	**114**	**164**	**194**

Continuation of Table 1 – Lipid and lipoprotein percentiles by gender and age group: 5th, 10th, 25th, 50th, 75th, 90th and 95th percentiles estimated for total cholesterol, LDL-C, HDL-C, TG, apoB, apoA1, sdLDL-C, Lp(a), non-HDL-C, apoB/apoA1, sdLDL-C/LDL C and remnant cholesterol.

ApoB		Percentile						
Sex	**Age group**	**5th**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**
	18-29	46	53	62	77	88	102	108
	30-39	62	66	80	101	115	127	136
Men	40-49	65	69	91	109	125	138	139
	50-59	71	77	93	103	126	144	151
	18-59	**55**	**62**	**77**	**93**	**110**	**127**	**142**
	18-29	51	54	68	77	88	101	120
	30-39	62	63	72	85	97	118	131
Women	40-49	63	67	75	90	100	121	136
	50-59	66	67	83	93	113	124	131
	18-59	**54**	**62**	**68**	**79**	**93**	**114**	**130**
Total	**18-59**	**56**	**63**	**74**	**88**	**103**	**118**	**130**

ApoA1		Percentile						
Sex	**Age group**	**5th**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**
	18-29	110	115	123	135	152	165	169
	30-39	105	114	123	139	152	174	187
Men	40-49	104	111	127	140	157	168	191
	50-59	116	117	129	148	165	183	194
	18-59	**109**	**116**	**123**	**135**	**155**	**167**	**182**
	18-29	123	130	145	168	188	203	216
	30-39	122	132	148	168	188	208	219
Women	40-49	115	129	141	158	185	195	200
	50-59	123	129	135	151	175	204	211
	18-59	**124**	**130**	**148**	**168**	**194**	**215**	**227**
Total	**18-59**	**110**	**117**	**128**	**147**	**167**	**191**	**208**

sdLDL-C		Percentile						
Sex	**Age group**	**5th**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**
	18-29	9	12	15	19	27	33	36
	30-39	14	17	21	29	39	51	62
Men	40-49	19	20	27	36	45	58	66
	50-59	16	20	24	33	50	64	72
	18-59	**13**	**15**	**18**	**27**	**37**	**51**	**61**
	18-29	9	11	15	20	30	41	44
	30-39	11	13	17	21	28	37	43
Women	40-49	13	15	19	23	31	42	46
	50-59	15	16	21	26	34	40	42
	18-59	**10**	**13**	**18**	**23**	**30**	**39**	**42**
Total	**18-59**	**12**	**14**	**18**	**25**	**33**	**44**	**53**

Lp(a)		Percentile						
Sex	**Age group**	**5th**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**
	18-29	3	3	6	12	22	49	64
	30-39	3	3	4	10	28	47	62
Men	40-49	3	3	5	11	20	49	62
	50-59	3	3	8	11	19	38	57
	18-59	**3**	**3**	**7**	**12**	**31**	**58**	**76**
	18-29	3	3	5	11	24	46	63
	30-39	3	3	4	10	16	44	52
Women	40-49	3	3	7	12	18	41	53
	50-59	3	3	8	12	35	64	69
	18-59	**3**	**3**	**5**	**11**	**20**	**41**	**64**
Total	**18-59**	**3**	**3**	**4**	**11**	**28**	**53**	**67**

Continuation of Table 1 – Lipid and lipoprotein percentiles by gender and age group: 5th, 10th, 25th, 50th, 75th, 90th and 95th percentiles estimated for total cholesterol, LDL-C, HDL-C, TG, apoB, apoA1, sdLDL-C, Lp(a), non-HDL-C, apoB/apoA1, sdLDL-C/LDL C and remnant cholesterol.

Non-HDL-C		Percentile						
Sex	**Age group**	**5th**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**
	18-29	70	77	93	114	138	155	167
	30-39	99	111	131	147	169	198	213
Men	40-49	108	111	135	163	185	198	226
	50-59	102	110	138	152	193	215	236
	18-59	**83**	**91**	**115**	**141**	**165**	**194**	**202**
	18-29	67	77	100	112	129	150	169
	30-39	83	90	106	123	145	161	177
Women	40-49	91	92	108	134	151	182	206
	50-59	88	105	122	146	166	189	204
	18-59	**77**	**90**	**100**	**117**	**140**	**167**	**190**
Total	**18-59**	**81**	**92**	**108**	**134**	**154**	**181**	**196**

ApoB/apoA1		Percentile						
Sex	**Age group**	**5th**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**
	18-29	0.32	0.38	0.44	0.54	0.68	0.79	0.88
	30-39	0.41	0.44	0.55	0.70	0.85	1.08	1.16
Men	40-49	0.46	0.50	0.61	0.76	0.93	1.17	1.24
	50-59	0.40	0.49	0.57	0.74	0.87	0.96	1.03
	18-59	**0.40**	**0.44**	**0.53**	**0.71**	**0.84**	**1.02**	**1.20**
	18-29	0.30	0.35	0.41	0.46	0.55	0.64	0.75
	30-39	0.31	0.36	0.44	0.51	0.60	0.67	0.71
Women	40-49	0.34	0.37	0.44	0.56	0.68	0.84	0.93
	50-59	0.34	0.37	0.46	0.62	0.79	0.92	0.98
	18-59	**0.31**	**0.36**	**0.42**	**0.51**	**0.62**	**0.76**	**0.85**
Total	**18-59**	**0.34**	**0.39**	**0.45**	**0.56**	**0.75**	**0.92**	**1.05**

sdLDL-C/LDL-C		Percentile						
Sex	**Age group**	**5th**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**
	18-29	0.14	0.15	0.17	0.22	0.26	0.33	0.37
	30-39	0.12	0.13	0.17	0.20	0.27	0.34	0.38
Men	40-49	0.11	0.14	0.17	0.21	0.27	0.36	0.41
	50-59	0.12	0.14	0.17	0.21	0.26	0.30	0.34
	18-59	**0.14**	**0.16**	**0.19**	**0.22**	**0.28**	**0.35**	**0.39**
	18-29	0.12	0.14	0.18	0.22	0.25	0.30	0.30
	30-39	0.11	0.14	0.18	0.21	0.26	0.28	0.39
Women	40-49	0.12	0.14	0.16	0.19	0.30	0.41	0.41
	50-59	0.12	0.13	0.15	0.20	0.27	0.34	0.37
	18-59	**0.12**	**0.14**	**0.18**	**0.21**	**0.25**	**0.29**	**0.33**
Total	**18-59**	**0.13**	**0.15**	**0.18**	**0.22**	**0.26**	**0.31**	**0.37**

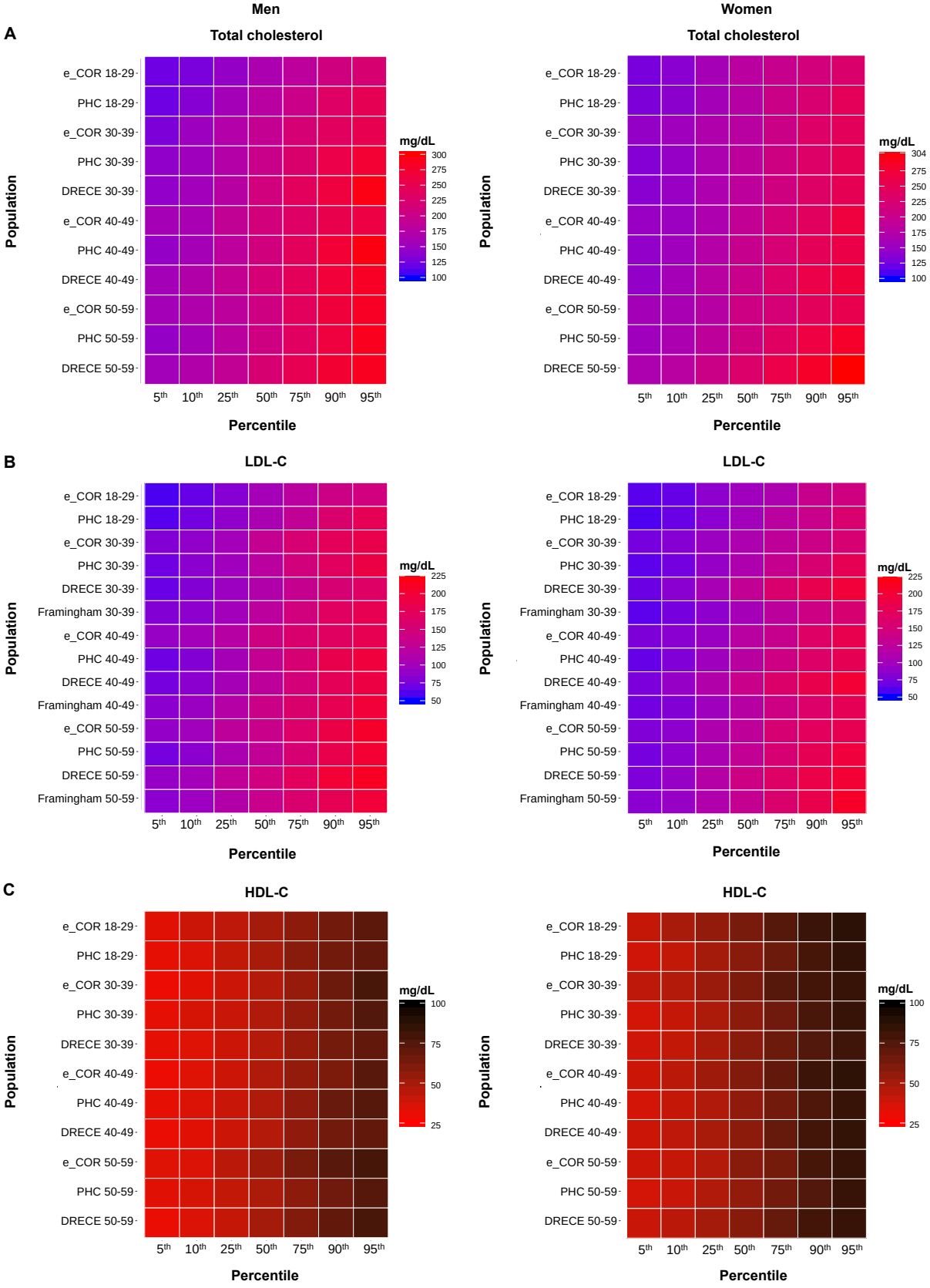
Remnant		Percentile						
Sex	**Age group**	**5th**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**
Men	18-29	5	6	9	14	21	26	30
	30-39	5	8	10	14	19	25	30
	40-49	4	7	10	17	22	29	36
	50-59	6	7	9	13	18	28	30
	18-59	**5**	**7**	**11**	**16**	**23**	**33**	**39**
Women	18-29	4	6	9	14	18	25	30
	30-39	6	7	10	15	21	27	31
	40-49	5	7	9	13	19	28	33
	50-59	5	8	9	15	21	23	30
	18-59	**5**	**7**	**10**	**14**	**20**	**25**	**29**
Total	**18-59**	**5**	**7**	**11**	**16**	**22**	**29**	**35**

Percentiles values are presented in mg/dL. LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1; Non-HDL-C, non-high-density lipoprotein cholesterol; sdLDL-C, small dense low-density lipoprotein cholesterol; Lp(a), lipoprotein (a); Remnant, remnant cholesterol.

3.2. Comparison of the lipid percentiles among different study populations

The 95% CI of the percentile values estimated for TC, LDL-C, HDL-C, TG, apoB, and apoA1 in the present study are described in Supplementary Tables 1-2. The percentile values estimated for the Portuguese population in the present study (e_COR Study) were compared with the percentile values estimated for the Portuguese primary health care users (PHC Study) in Portugal in 2013 (Cortez-Dias et al., 2013), and also with the Spanish (DRECE Study) (Gómez-Gerique et al., 1999) and American populations (Framingham Offspring Study and the NHANES III) (Contois et al., 1996b; Bachorik et al., 1997) (Figure 1 and Supplementary Figures 3-18).

The graphs show similar patterns for the lipid biomarkers between e_COR and other study populations, although differences in percentile values were seen (Figure 1 and Supplementary Figures 3-18). The TC and TG were predominantly higher in the PHC Study, while the highest LDL-C and HDL-C was observed in men and women of the e_COR Study, respectively. When comparing the e_COR and DRECE studies, results showed that TC, HDL-C and TG percentile values were predominantly higher in almost all age groups for both men and women of the Spanish population, while the LDL-C were predominantly lower in men up to 50. The comparison of the LDL-C percentile values between the e_COR and Framingham Offspring Studies showed no marked differences for both men and women, but the Framingham Offspring Study showed slightly lower values for almost all percentiles. Looking at apoB values, the percentiles were predominantly lower in the Framingham Offspring Study for men and women aged 30-39, but this difference was reversed in the highest percentiles (90^{th} and 95^{th}) of women aged 50-59. Comparing apolipoprotein percentiles between e_COR and the other American population, NHANES III, apoB values were predominantly higher for all age groups of men and women of the NHANES III population, with a more marked increase of the percentile levels from age 50. For apoA1, percentile values were predominantly lower in men for all age groups of the NHANES III population. In women, these values were significantly lower in age group 30-39 and for almost all percentiles of the age group 40-49, with this difference disappearing from age 50.


A
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PHC 30-39
DRECE 30-39
Framingham 30-39
e_COR 40-49
PHC 40-49
DRECE 40-49
Framingham 40-49
e_COR 50-59
PHC 50-59
DRECE 50-59
Framingham 50-59
5th 10th 25th 50th 75th 90th 95th
Percentile
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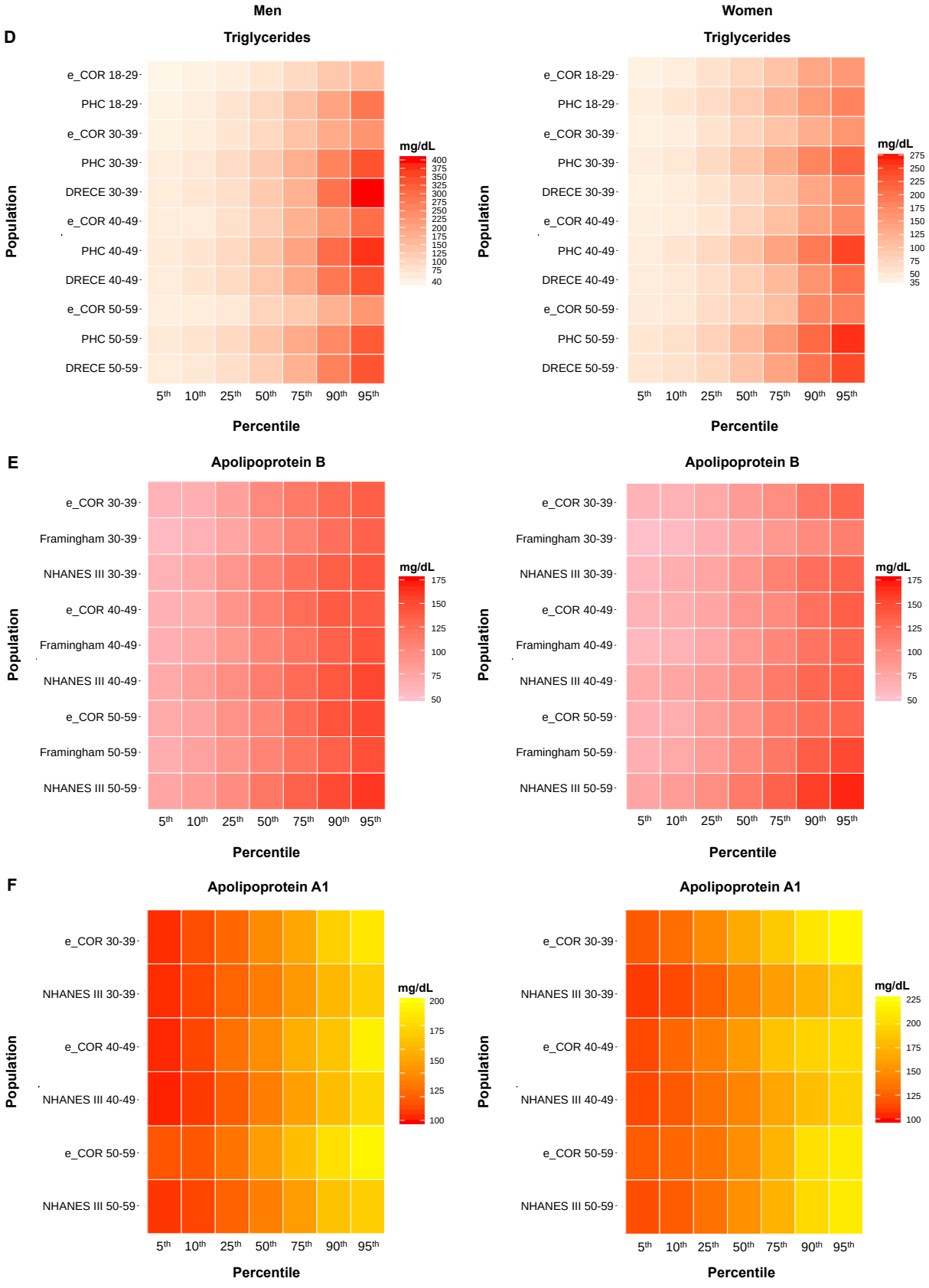
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PHC 50-59
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Population
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5th 10th 25th 50th 75th 90th 95th
Percentile
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Framingham 30-39
NHANES III 30-39
Framingham 40-49
NHANES III 40-49
Framingham 50-59
NHANES III 50-59
F
Apolipoprotein A1

Figure 1 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles between different populations for total cholesterol (TC) (**A**), LDL-C (**B**), HDL-C (**C**), triglycerides (TG) (**D**), apolipoprotein B (ApoB) (**E**), and apolipoptrotein A1 (ApoA1) (**F**). The percentiles estimated for the Portuguese population in the present study (e_COR Study) were compared with the percentiles estimated for the primary health care (PHC) users in Portugal in 2013, with the Spanish population (DRECE Study), and also with the percentiles estimated for the American populations (National Health and Nutrition Examination Survey III – NHANES III, and Framingham Offspring Study) for both men (left) and women (right). In each graph, the study name is followed by age group. The percentile values are in mg/dL, represented by a colour gradient with values ranging from 100 to 300 (men) and 100 to 304 (women) for TC, 50 to 225 (men and women) for LDL-C, 25 to 100 (men and women) for HDL-C, 40 to 400 (men) and 35 to 275 (women) for TG, 50 to 175 (men and women) for apoB, and from 100 to 200 (men) and 100 to 225 (women) for apoA1, respectively. The plot graphs using the 95% confidence intervals (CI) percentiles estimated for the Portuguese population are provided in Supplementary Figures 3-18.

For sdLDL-C and remnant cholesterol, it was not possible to compare results since the other studies do not have percentiles for these biomarkers. For Lp(a), there is another analysis of the Framingham Offspring Study (Jenner et al., 1993; Contois et al., 1996a; Seman et al., 1999; Lamon-Fava et al., 2011) that presented percentile values, but it was not possible to compare results since these studies do not have the same age groups considered in this analysis and/or there were relevant discrepancies between results. A study from Nordestgaard and colleagues (Nordestgaard et al., 2010) also presented Lp(a) percentile values, and although it was not possible to compare results, in this case our values seem to be lower: the ~50 mg/dL value for the overall population corresponds to the 80th percentile (P80th) of the Nordestgaard *et al*. Study and to the P90th of the e_COR Study.

4. DISCUSSION

Dyslipidaemia is one of the major cardiovascular risk factors and population-specific reference intervals are of great value for its evaluation. Here, we proposed for the first time reference values for plasma TC, LDL-C, HDL-C, TG, apoA1, apoB, sdLDL-C, Lp(a), as well as for non-HDL-C, apoB/apoA1 and sdLDL-C/LDL-C ratios, and remnant cholesterol, for the Portuguese population, based on lipid percentiles. All percentiles were estimated using a bootstrap methodology, a valid tool for percentile determination, and taking into account gender and age-specific stratum weights, which were used to overcome the limitation of the e_COR sample not being representative of our population due to the study design. This way, the values obtained are representative of the Portuguese population. These newly determined reference values for lipid biomarkers will allow a correct dyslipidaemia assessment and the use of these reference values in the clinic, for a better patient care and management. Until now, the only percentiles for the Portuguese population were determined for primary care users and not for the general population, and only included values for TC, LDL-C, HDL-C and TG (Cortez-Dias et al., 2013). Thus, we believe that the percentiles presented here for both men and women will reflect better the Portuguese reality and should therefore be used as references for our adult population. Being sdLDL an important atherogenic biomarker, it is to note that, to our knowledge, the percentiles for sdLDL-C are the first to be established for a population in Europe and could be of use to other populations, at least in southern European populations with similar lifestyles.

Interesting to note is that our P50th cut-off points for TC and LDL-C are very similar to the ESC/EAS recommendation (Catapano et al. 2016), the TC P50th

being 189 mg/dL (ESC/EAS 190 mg/dL) and LDL-C P50th being 116 mg/dL (ESC/EAS 115 mg/dL). For HDL-C and apoA1, our cut-off points are also not very different from what is considered as at risk according to the ESC/EAS guidelines (Catapano et al. 2016), the P10th being 34 mg/dL for men (ESC/EAS 40 mg/dL (Catapano et al. 2016) and 45 mg/dL for women (ESC/EAS 48 mg/dL (Catapano et al. 2016), and the apoA1 P10th being 116 mg/dL for men (ESC/EAS 120 mg/dL (Catapano et al. 2016) and 130 mg/dL for women (ESC/EAS 140 mg/dL). As expected, with TG levels being very depended on diet, TG concentration was very variable in our sampled population, and also when comparing with the PHC study (Cortez-Dias et al., 2013). In the case of apoB, there are no defined cut-off points by ESC/EAS guidelines, but the American Association of Clinical Endocrinologists (Jellinger et al., 2016) (AACE) recommend an optimal apoB value below 90 mg/dL, which is very similar to our P50th (88 mg/dL). For the Lp(a), whose reference values have been subject of debate, our P90th is 53 mg/dL, similar to the defined at risk value according to the 2016 ESC/EAS guidelines (Catapano et al. 2016), although in these guidelines this value is the P80th. However, it is important to point out that our values are generally lower than the values described for other populations (Kamstrup et al., 2008, 2013; Nordestgaard et al., 2010; Marcovina and Albers, 2016; Nordestgaard and Langsted, 2016); since Lp(a) is mainly determined genetically (Sandholzer et al., 1991; Kamstrup et al., 2009, 2013; Tsimikas, 2017) comparisons of this biomarker may not be accurate.

In this study, it was also proposed to compare these percentiles with the percentiles from other population studies, for comparison between populations. Primary health care users in Portugal (2013) (Cortez-Dias et al., 2013), and also from a Spanish population (DRECE Study (Gómez-Gerique et al., 1999)) and American populations (Framingham Offspring Study (Contois et al., 1996b) and the NHANES III Study (Bachorik et al., 1997), were compared by plotting the percentile graphs from each study together with the estimated percentiles and corresponding estimated 95% CI from the e_COR Study, by gender and age group. Hence, we showed a feasible method to compare percentile values, using a very graphic method that allows a good visualization of differences and similarities. Although the bootstrapping methodology is not a new method, we took the advantage of the percentiles estimation by calculating the 95% CI at the same time, and applied these values in the construction of plot graphs using the R software (R: The R Project for Statistical Computing 2017). To our knowledge, this study is the first to compare the lipid percentiles among populations, using graphs constructed with colour scales and also with the 95% CI.

Several differences for all lipid parameters were noted when comparing the percentiles estimated in this study and a different study from Portuguese population (PHC). Although expected this highlights the usual problems due to differences in strategy design and populations: e_COR is a national study and the PHC only comprised individuals from primary health care. Also, in the e_COR Study all biochemical parameters were determined by a single central laboratory, under the same conditions with a fasting period of 12 hours, and in the PHC Study biochemical values were taken from patient files. These differences seen, especially in TG values, adds evidence that 12 hours fasting is important to accurately measure lipid concentrations, and that for population studies, the use of a single laboratory is important for lipid profile determination.

Interestingly, similar to that observed between e_COR and the PHC, despite the close geographic proximity between Portugal and Spain, large differences in the TG concentrations were observed between them. This should not be surprising due to, again differences in methodology, but also since TG is influenced by diet and alcohol intake (Mensink et al., 2003; Stanhope et al., 2009; Kelishadi et al., 2014). Additionally, as lipid parameters could be modulated by environmental factors and other non-genetic and genetic factors, differences should be expected at some level.

Although differences were seen between e_COR and the NHANES III, e_COR presenting lower values for apoB and higher values for apoA1, no marked differences were observed for the LDL-C and apoB percentiles between e_COR and the Framingham Offspring populations; a large difference was expected since this comparison is between a south European and an American population known to have a completely different life habits specially on the diet concerning fat intake. However, we have to take into account that our study did not include individuals under lipid-lowering therapy in the estimation of the percentiles, which could be a possible explanation for this small difference between such distinct populations; it is not clear if Framingham individuals were under medication. Still, it is also important to note that our LDL-C values were determined by direct assay techniques and not using the Friedewald equation; discrepancies among results were noted in previous studies that have investigated the difference between methods (Sibal et al., 2010; Martin et al., 2013; Balder et al., 2017).

Taking all these results into consideration, we recommend the newly determined lipid percentiles of the Portuguese population to be used in a clinical context, since they were obtained by a rigorous and powerful methodology. It is important to note that these values were estimated based on what could be considered a general population with untreated lipid values. Using these percentile values as reference, will provide a picture of how deviated an individual patient's value is from the expected in the global population.

The P50th can be considered optimal, above the P90th for TC, LDL-C, TG, apoB, sdLDL-C, Lp(a), non-HDL-C apoB/apoA1 and remnant cholesterol, or below the P10th for HDL-C and apoA1 can be considered at risk. High risk can be defined above the 95th or below the 5th percentiles, and so it can also be defined as the cut off for the different lipid disorders. These percentiles can be useful to select individuals with extreme dyslipidaemia phenotypes to further investigate the association between extreme lipid values and variants in genes associated with plasma lipids and lipoproteins. In fact, the identification of new variants in individuals with extremely low or high plasma lipid levels has been successful (Cohen et al., 2004; Patel et al., 2016). With the new era of next-generation sequencing, the massively high throughput sequencing data which allows the analysis of several genes at the same time, this is feasible and much more cost-effective, and can add evidence to genotype and phenotype relationships in the field of lipid disorders.

In addition, it is worth mentioning that bootstrapping used here was an advantageous methodology, taking into consideration that in terms of distribution by age and gender the collected samples were not representative of Portuguese population, as well as that the data did not assume a particular Gaussian probability distribution (Efron, 1979; Henderson, 2005). Wherever data from a population are not representative, we recommend this strategy to be used.

5. STUDY LIMITATIONS

Although a high number of individuals were included in the estimation of lipid reference values, the number of individuals included is smaller than overall sample, especially for the age group 50-59, due to exclusion of cases with secondary causes of dyslipidaemia and/or under lipid-lowering therapy (51.30%, n=866). However, we believe that applying these exclusion factors was the right decision, since the objective was to determine reference values based on what could be considered a general population with untreated lipid values, with applicability to the clinical community. Additionally, the extensive exploratory data analysis applied here and the applicability of the methodology was advantageous, since it produced gains in precision. Another limitation was the inclusion of women under hormonal therapy, which is known to affect slightly the lipid metabolism (Bachorik et al., 1997), since they represented 20% (n=341) of the overall sample. Also, the participants of the e_COR Study were almost exclusively Caucasians (98%). Unfortunately, it was also not possible to conduct the comparison analysis of percentiles for all age groups and lipid biomarkers, due to the differences in the study design between populations and lack of information. Indeed, other limitations should be

considered in extrapolating results due to the method of assessing lipid levels and also to the differences in the data of the studies, since modifications in age and lifestyle could be time-dependent.

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7. SUPPLEMENTARY DATA

7.1. Supplementary Material and Methods

7.1.2. Study type

The e_COR Study (study of the prevalence of cardiovascular risk factors of the Portuguese population) was designed as an observational cross-sectional epidemiological study (Bourbon et al., 2018).

The study was previously approved by the National Commission for Data Protection and the National Institute of Health (INSA) Ethical Committee.

7.1.3. Sample definition

The aim was to have a stratified proportional sample with representation of genders, all Portuguese continental regions and ages pre-defined to be ≥18 years and <80 years. The database used for the sample was the National Register of users (RNU) (National Register of Users, 2017) in 2011 – individuals aged ≥18 years and <80 years. It was defined as a sample with 1,685 individuals, distributed equally by the five continental regions (NUTS II), based on the following assumptions:

The determined minimum sample size, with national representation required to determine the prevalence of cardiovascular risk factors in the Portuguese population was 1,040 individuals, based on population data from the *Instituto Nacional de Estatística* (INE) (Instituto Nacional de Estatistica, Censos 2011), and taking into account the prevalence of hypertension (HT) determined by the study "Prevalence, knowledge, treatment and Control of Hypertension in Portugal" (PAP Study) (De Macedo et al., 2017), which was 42% and a sampling error of 3%.

A random sampling method that involved three levels: 1) simple random selection of two health centre groups (ACES) (Cuidados de Saúde Primários, 2017) for each of the five continental health regions; 2) simple random selection of two health centres (CS) for each ACES; 3) simple random selection of participants registered in each chosen CS, weighted by the proportional size of users of each CS within the ACES and divided equally by the three defined age groups. The data of users of each CS were kindly provided by the Central Administration of the Health System (ACSS) (ACSS, 2017) in 2011.

The defined exclusion criteria were pregnant or postpartum women up to 3 months; inability to understand/speak Portuguese; declared mental disease; residence outside the

study region; telephone contact failure after 3 different attempts at different days and hours.

A total of 1,688 unrelated adults, 848 men and 840 women aged between 18 and 79, and recruited from the Norte, Centro, Lisboa, Alentejo and Algarve regions were included in the e_COR Study. The rate of respondents who entered the e_COR Study was 34%, with the remaining 76% not interested in taking part in our survey, the majority due to lack of time/availability.

7.1.4. Data collection

The data and sample collection of each participant was processed sequentially (with the signature of informed consent always being the initial step), and consisted of the following: (**1**) read and signed informed consent; (**2**) fasting venous blood sample collection for analysis of biochemical parameters; (**3**) physical examination (blood pressure, weight, height and waist circumference measurements); (**4**) questioning based on the study questionnaire formulated by a team member. Detailed description of the 4 study steps are described below.

Step 1 – Informed consent: all participants were properly informed about the study, and had the opportunity to discuss all matters considered relevant on it, before starting their participation. After clarification, all subjects signed informed consent to accept their participation in the study;

Step 2 – Blood collection: the blood sample was obtained after fasting for about 12 hours. For each participant approximately 16 mL of blood was withdrawn for the determination of biochemical parameters, as well as for DNA extraction (1 serum gel tube 7.5 mL, 3 EDTA tubes 2.7 ml);

Step 3 – Physical examination: the physical examination consisted of measurement blood pressure, systolic (SBP) and diastolic (DBP), weight, and height and waist circumference. The blood pressure measurement was performed in the sitting position after at least 10 minutes rest with a digital sphygmomanometer (M6 Comfort, OMRON), and one measurement was performed on the left arm and two on the right arm. The value used in the data analysis was the arithmetic average of these three measurements. Physical examination also included the determination of weight and height (digital scale SEC-899 and SEC-217 stadiometer, CEAS), with the participant using only light clothes and no shoes. However, for calculating the body mass index (BMI) 0.5 kg was removed. The measurement of waist circumference was held at the midpoint between the lower edge of

the last rib and the iliac crest (flexible tape SEC-201, SECA), with the participant standing, wherever possible;

Step 4 – Questionnaire application: the questionnaire was developed by the study team and was divided into 10 main sections: personal data; recent clinical information; medication; information on high cholesterol, high triglycerides (TG); information on diabetes and HT; chronic diseases; smoking habits; eating habits; physical activity. The form consists of questions with open and closed response. The women were also asked about the use of birth control pills and/or hormone therapy, number of pregnancies, number of miscarriages and age of menopause.

7.1.5. Sample Processing

After collection, the blood was maintained at rest for between 30 minutes and 3 hours, and then centrifuged at 3,000 rpm for 15 minutes. After centrifugation, the serum and plasma were stored with temperature controlled between 2 °C and 8 °C, then was transported in a refrigerated environment to INSA, where the samples were processed within a period of 36 hours. All biochemical determinations were performed at the Diagnosis and Reference Laboratory Unit (UDR) of INSA, in Lisbon or Porto.

Biomarkers for the metabolism of glucose were determined for 1,676 and 1,688 individuals, respectively, in a Cobas Integra 400 plus (Roche, Risch-Rotkreuz, Switzerland) by enzymatic colorimetric and immunoturbidimetric methods, using the hexokinase enzyme. The biochemical tests for total cholesterol (TC), direct low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), TG, apolipoprotein A1 (apoA1), and apolipoprotein B (apoB) were determined for all 1,688 samples in an autoanalyser Cobas Integra 400 plus (Roche, Risch-Rotkreuz, Switzerland), also by an enzymatic colorimetric and immunoturbidimetric method. Serum levels of small, dense low-density lipoprotein cholesterol (sdLDL-C) were measured in 1,669 samples (98.87%) by direct quantification in an autoanalyser RX Daytona (Randox Laboratories, Crumlin, United Kingdom) by an enzymatic colorimetric method (sLDL-EX ”Seiken”), while lipoprotein(a) [Lp(a)] was measured in 895 samples (53.02%) by immunoturbidimetric methods.

7.1.6. Definition of study variables considered in the present study

The variables of the e_COR Study that were considered for the data analysis of the present study are described below.

7.1.6.1. Diabetes

It was considered that an individual had diabetes when the level of fasting glucose was greater than 126 mg/dL (determined on two separate occasions) or below that threshold, when under therapy for diabetes; presenting pre-diabetes when the glucose level was between 110 mg/dL and 126 mg/dL (Cuidados de Saúde Primários, 2017; ACSS, 2017).

7.1.6.2. Hypothyroidism/ hyperthyroidism

It is considered that an individual had hypothyroidism or hyperthyroidism if they said they were diagnosed or if they were or under treatment for hypothyroidism or hyperthyroidism.

7.1.6.3. Lipid-lowering therapy

Individuals reporting any lipid-lowering therapy for cholesterol and/or triglycerides at the time blood collection were considered under lipid-lowering therapy.

7.2. Supplementary Tables

7.2.1. Supplementary Table 1

Supplementary Table 1 – Confidence intervals (95%) for total cholesterol, LDL-C, HDL-C, triglycerides, apolipoprotein B, and apolipoprotein A1 percentiles for men of the e_COR Study population.

		95% confidence intervals						
Lipid biomarker	**Age group**	5th	10th	25th	50th	75th	90th	95th
Total cholesterol	18-29	118-129	123-135	137-155	161-172	178-195	203-219	211-231
	30-39	130-143	141-160	165-177	193-206	221-234	239-250	250-278
	40-49	147-163	160-165	178-195	207-217	222-236	250-260	262-274
	50-59	150-175	157-179	176-190	191-222	223-260	251-293	262-309
LDL-C	18-29	56-67	61-75	76-90	96-107	114-130	131-146	140-152
	30-39	72-87	80-93	94-114	127-137	146-159	168-179	175-193
	40-49	87-101	92-104	107-122	136-152	153-167	167-180	173-188
	50-59	64-104	89-113	105-130	130-149	147-181	172-309	182-232
HDL-C	18-29	32-33	33-40	43-46	50-51	56-59	64-72	72-77
	30-39	28-33	31-36	38-41	44-49	52-58	59-76	68-85
	40-49	23-34	30-36	36-42	43-51	52-57	57-73	61-96
	50-59	33-38	34-41	38-49	49-57	57-67	65-83	68-87
Triglycerides	18-29	31-46	41-50	50-60	64-74	83-108	110-144	130-226
	30-39	45-53	49-61	65-76	88-108	121-156	168-222	188-266
	40-49	50-60	56-68	64-94	98-141	149-195	183-294	218-333
	50-59	50-60	51-63	61-81	84-113	114-155	136-241	167-255
ApoB	18-29	42-53	47-57	58-65	72-81	83-95	97-108	103-120
	30-39	56-66	62-68	73-87	98-104	110-119	126-131	129-144
	40-49	65-69	65-84	85-97	104-112	113-129	133-139	138-158
	50-59	61-81	65-88	84-99	101-109	110-134	131-152	136-163
ApoA1	18-29	101-114	110-119	120-127	132-141	144-156	157-168	165-184
	30-39	97-113	110-116	119-126	135-141	148-158	162-179	178-188
	40-49	94-11	104-122	121-130	132-148	149-163	161-191	167-214
	50-59	104-122	111-126	123-139	140-158	159-176	170-198	177-213

LDL-C, low-density lipoprotein cholesterol; HLDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1.

7.2.2. Supplementary Table 2

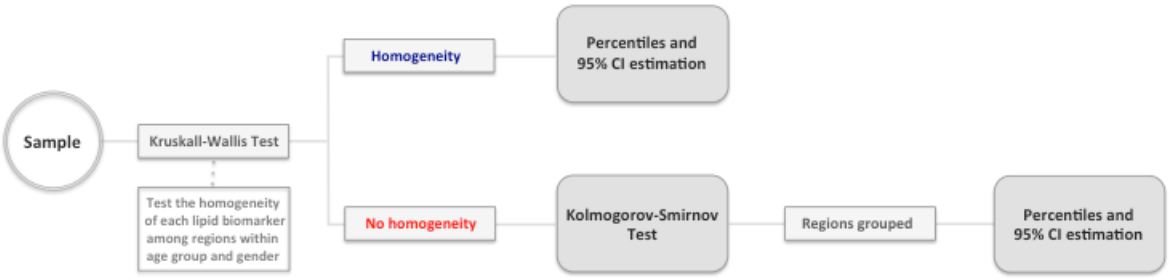
Supplementary Table 2 – Confidence intervals (95%) for total cholesterol, LDL-C, HDL-C, triglycerides, apolipoprotein B, and apolipoprotein A1 percentiles for women of the e_COR Study population.

		95% confidence intervals						
Lipid biomarker	**Age group**	5th	10th	25th	50th	75th	90th	95th
Total cholesterol	18-29	18-29	124-139	134-153	157-167	180-187	195-203	212-219
	30-39	30-39	140-156	153-158	171-174	180-200	196-214	214-225
	40-49	40-49	149-157	151-164	162-180	183-200	207-224	221-268
	50-59	50-59	146-170	156-173	172-199	200-214	214-240	225-257
LDL-C	18-29	18-29	54-66	66-78	84,7-905	97-101	105-110	125-143
	30-39	30-39	69-81	75-86	89-98	104-113	122-132	136-153
	40-49	40-49	71-83	76-90	86-108	113-125	128-144	139-183
	50-59	50-59	68-90	79-102	94-118	118-140	140-164	158-183
HDL-C	18-29	18-29	36-48	44-53	53-57	61-67	71-77	79-87
	30-39	30-39	40-48	43-49	51-56	50-66	71-76	77-82
	40-49	40-49	38-46	39-50	49-56	57-62	63-75	73-87
	50-59	50-59	36-41	38-45	42-51	52-60	60-76	74-87
Triglycerides	18-29	18-29	30-44	40-50	54-64	71-84	94-110	119-149
	30-39	30-39	37-44	42-50	54-67	75-84	97-113	121-145
	40-49	40-49	39-47	43-48	48-65	68-87	95-124	122-178
	50-59	50-59	46-57	47-63	58-75	76-92	92-134	116-190
ApoB	18-29	18-29	49-52	51-61	64-69	75-79	83-93	97-120
	30-39	30-39	50-63	62-66	70-75	80-88	92-104	109-130
	40-49	40-49	60-67	62-70	69-85	87-93	95-108	103-137
	50-59	50-59	53-68	57-80	70-85	85-105	106-119	117-132
ApoA1	18-29	18-29	113-129	124-136	139-153	163-175	181-194	197-213
	30-39	30-39	115-131	123-138	142-154	162-171	182-195	197-217
	40-49	40-49	115-129	115-133	139-143	152-164	180-190	195-200
	50-59	50-59	110-130	119-133	131-144	145-164	165-194	185-212

LDL-C, low-density lipoprotein cholesterol; HLDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1.

7.3. Supplementary Figures

7.3.1. Supplementary Figure 1



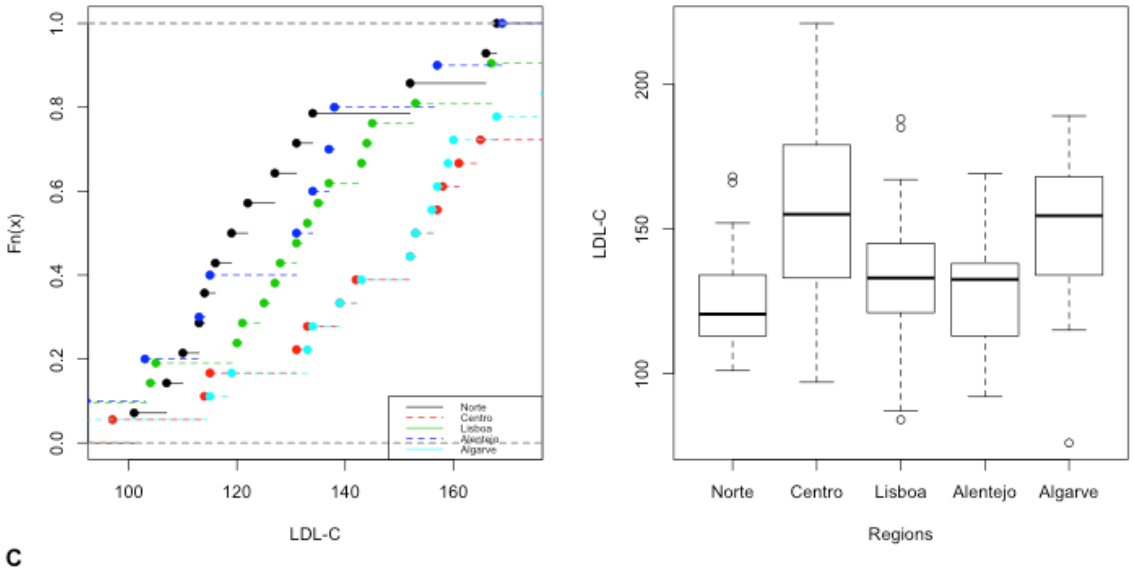
Supplementary Figure 1 – Schematic representation of testing the homogeneity distribution of lipid parameters among regions. The homogeneity of the distribution of lipid parameters among regions was tested within each age group and gender using Kruskal-Wallis non-parametric statistical test. For the age groups with evidence of lack of homogeneity, a Kolmogorov-Smirnov test was applied between regions to assess lack of homogeneity among pairs of regions. Regions for which the homogeneity hypothesis was not rejected were grouped and analysed as one individual stratum. Considering for each region/group of homogeneous regions, the respective stratum weights percentiles and 95% confidence intervals (CI) were estimated by bootstrapping.

7.3.2. Supplementary Figure 2

A

	Gender	Age group	Region	P value				
				Norte	Centro	Lisboa	Alentejo	Algarve
Men			Norte	–	0.034*	0.397	0.882	0.018*
40-49			Centro	–	–	0.135	0.060	0.964
P= 0.013*	Men	40-49	Lisboa	–	–	–	0.927	0.135
			Alentejo	–	–	–	–	0.060
			Algarve	–	–	–	–	–

B

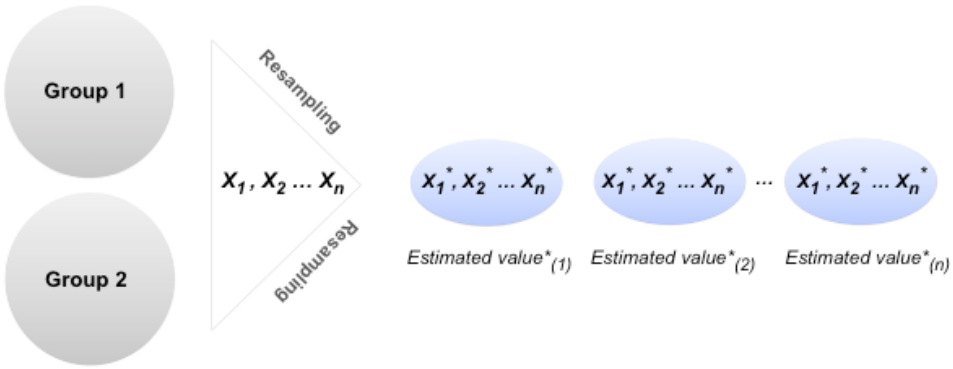


C

Regions grouped by clusters	Group 1	Group 2
	Norte	Centro
	Lisboa	Algarve
	Alentejo	

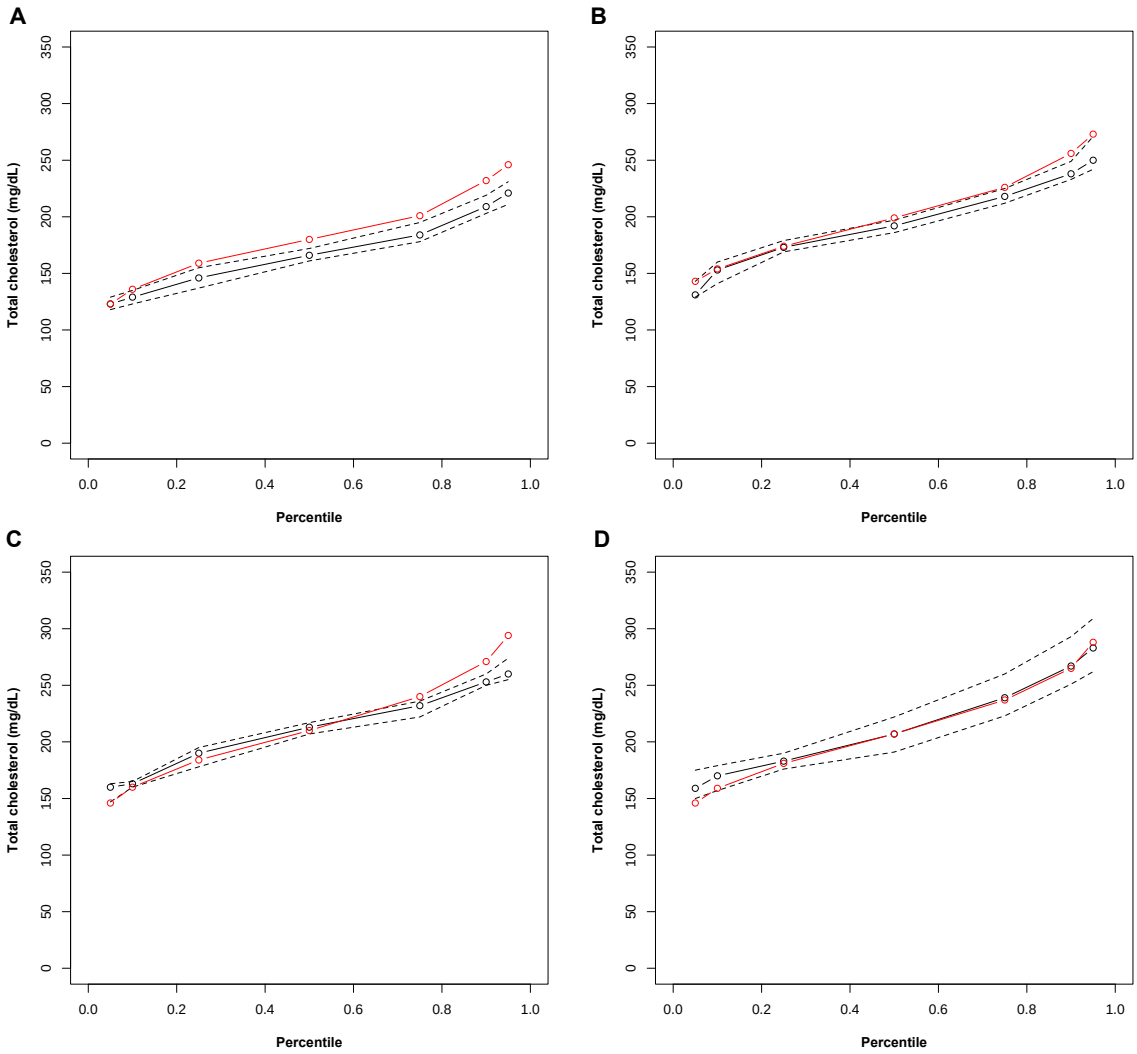
Resampled 50,000 times with distinct number of bootstrap samples according to the stratum weight of each group

D



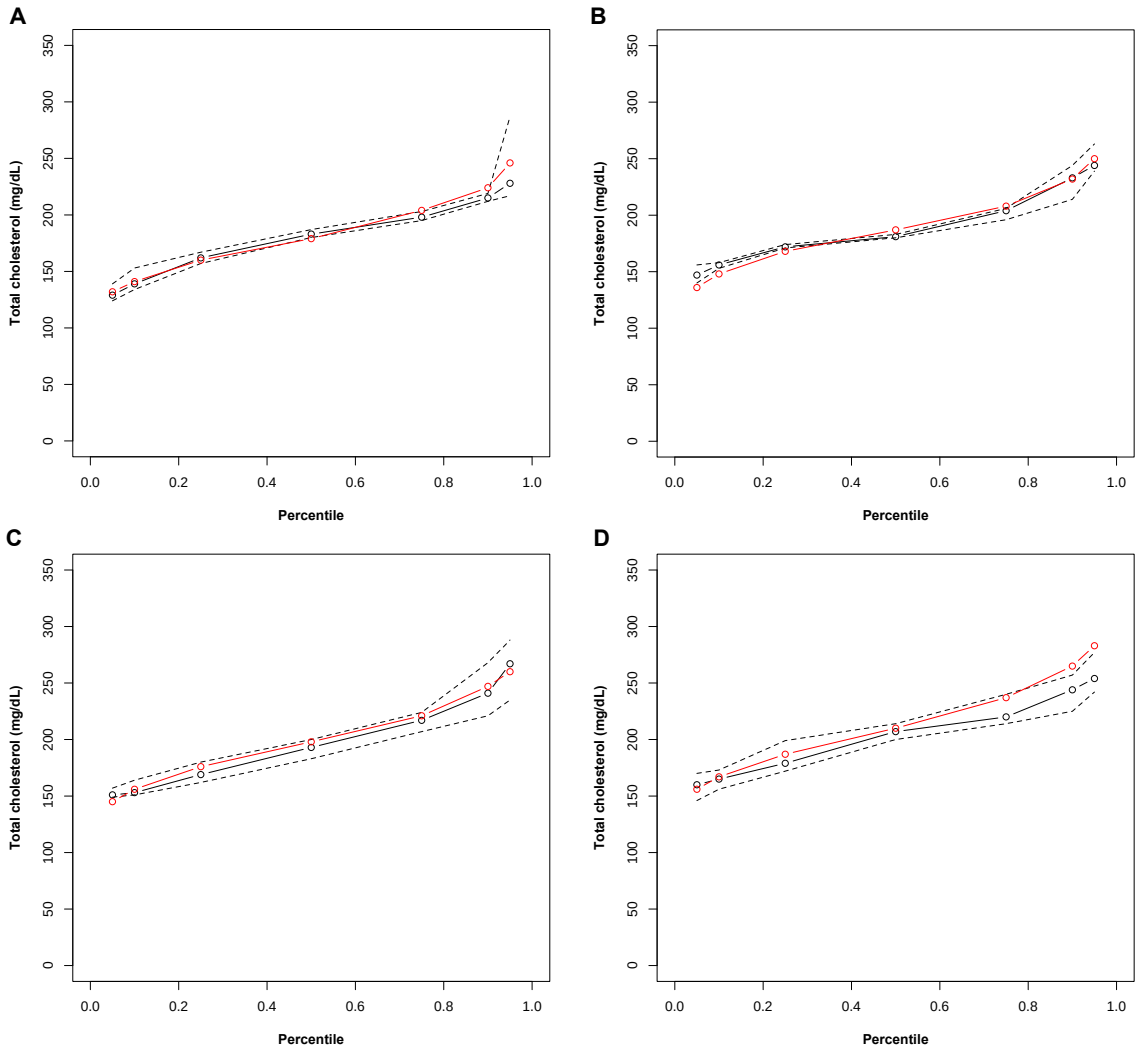
Supplementary Figure 2 – The percentiles for all lipid biomarkers were calculated taking into consideration that our sample was not representative by gender and age. The strategy was to assess whether the global sample could be considered a representative sample of the Portuguese population for each biomarker, so deviations from homogeneity were tested. As an example, for the LDL-C in men, the distribution of lipid biomarkers among regions was tested for men within each age group and gender using Kruskal-Wallis non-parametric statistical test. (**A**) The age group 40-49 showed evidence of lack of homogeneity, so a Kolmogorov-Smirnov non-parametric test was applied between regions to assess lack of homogeneity among pairs of regions. (**B**) Empirical cumulative distribution function (ECDF) plot and Box plot graphs were constructed to complement the analysis. (**C**) Regions presenting more homogeneity were grouped and analysed as one individual stratum (taking the respective stratum weights into account), and percentiles for these cases were calculated by bootstrapping. (**D**) In bootstrap, a set of (*n*) values are randomly resampled with replacement and this is repeated many times, each time producing a bootstrap estimated value. In our case data were resampled 50.000 times and the number of distinct bootstrap samples determined according to the stratum weights. The resampling was performed using different number of values, so in the end the estimated values were selected from a number that neither overestimates and nor underestimates the samples from each group.

7.3.3. Supplementary Figure 3



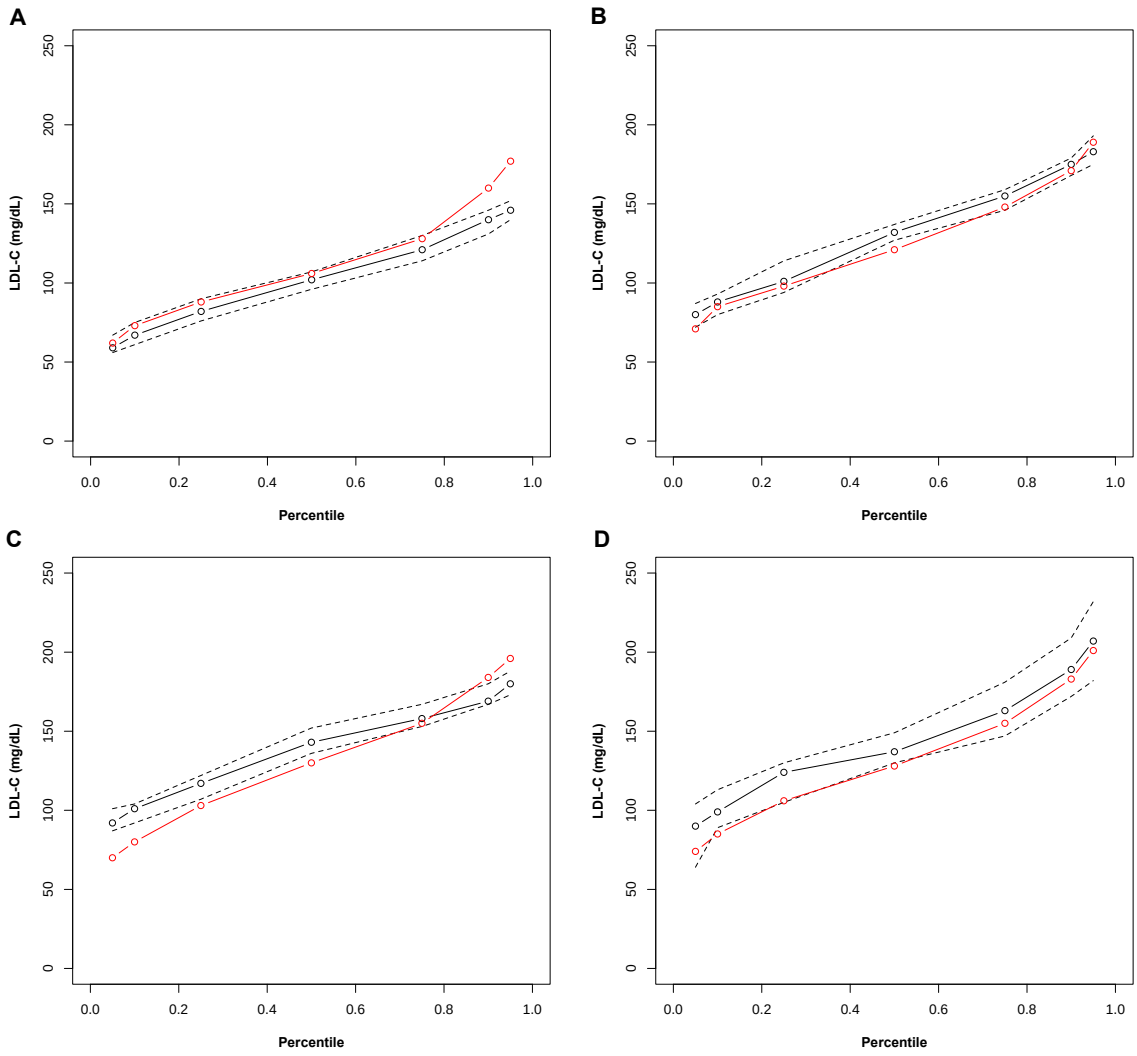
Supplementary Figure 3 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the total cholesterol (TC) in men, between e_COR Study and the Primary health care (PHC) users Study in Portugal, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the primary health care (PHC) users in Portugal in 2013 in men age groups 18-29 (**A**), 30-39 (**B**), 40-49 (**C**), and 50-59 (**D**). The TC percentiles values are in mg/dL, represented by black (e_COR) and red (PHC) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for TC is 0 to 350. Scale for percentiles is 0 to 1.0.

7.3.4. Supplementary Figure 4



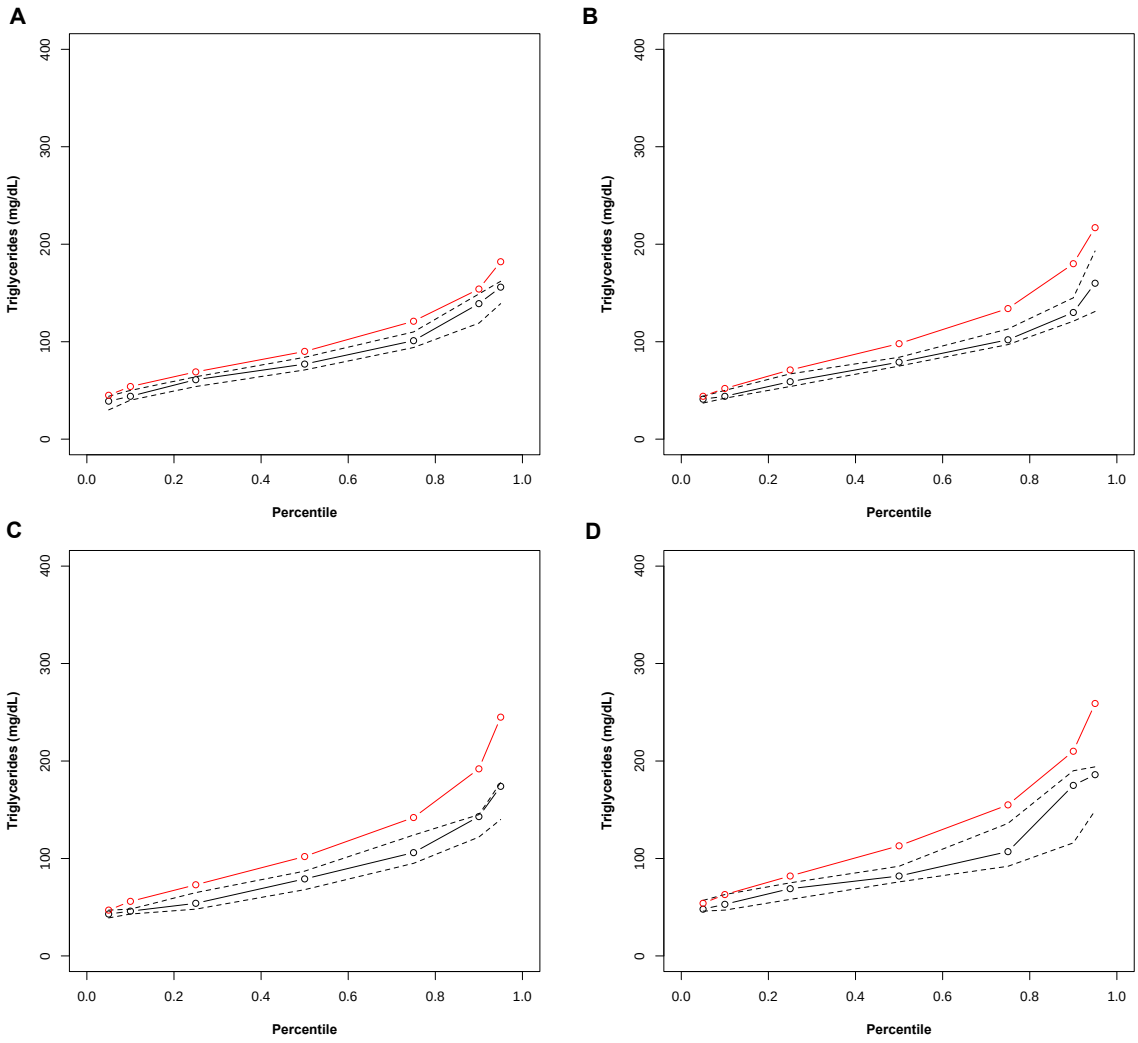
Supplementary Figure 4 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the total cholesterol (TC) in women, between e_COR Study and the Primary health care (PHC) users Study in Portugal, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the primary health care (PHC) users in Portugal in 2013 in women age groups 18-29 (**A**), 30-39 (**B**), 40-49 (**C**), and 50-59 (**D**). The TC percentiles values are in mg/dL, represented by black (e_COR) and red (PHC) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for TC is 0 to 350. Scale for percentiles is 0 to 1.0.

7.3.5. Supplementary Figure 5



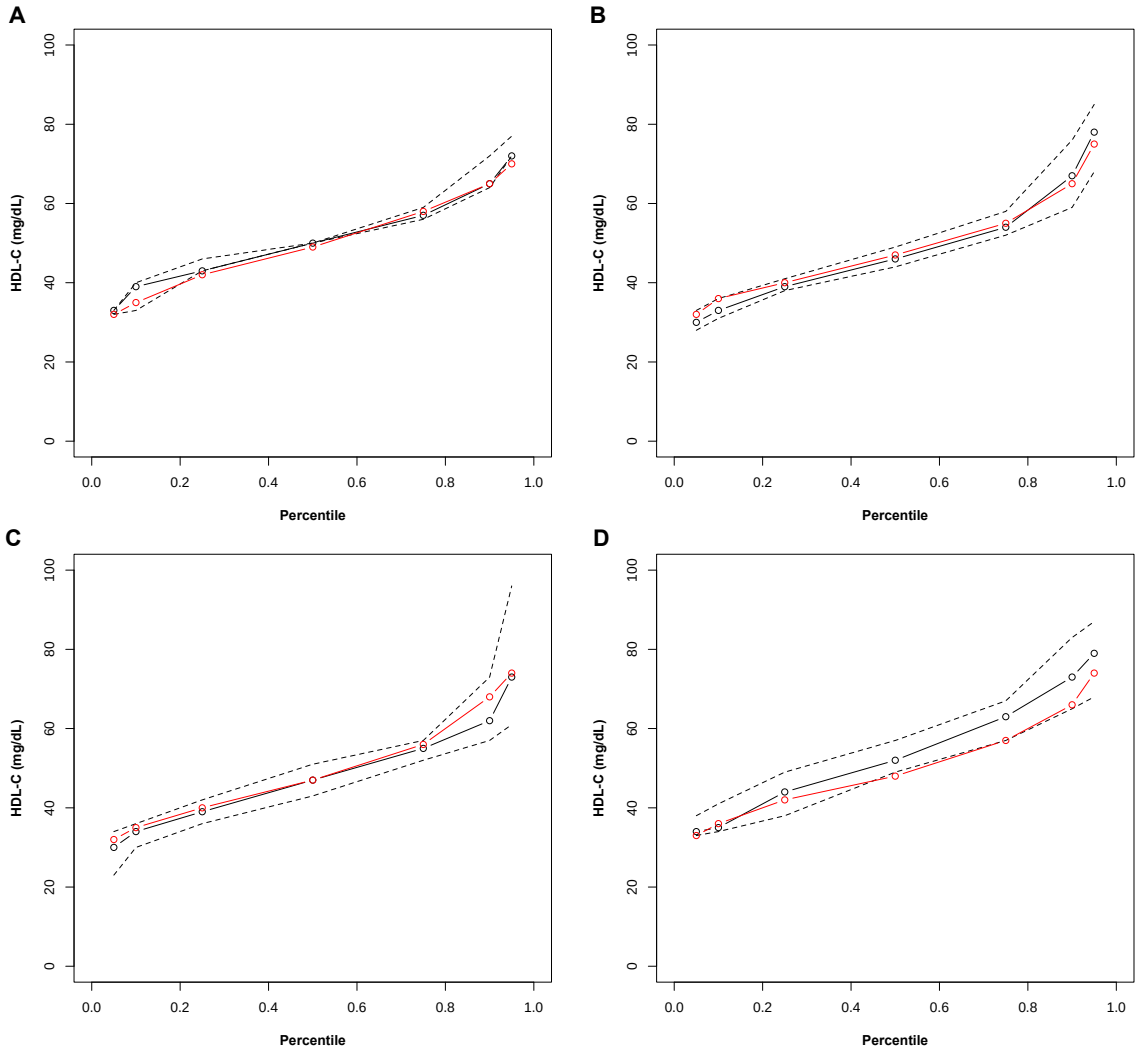
Supplementary Figure 5 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the LDL-C in men, between e_COR Study and the Primary health care (PHC) users Study in Portugal, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the primary health care (PHC) users in Portugal in 2013 in men age groups 18-29 (**A**), 30-39 (**B**), 40-49 (**C**), and 50-59 (**D**). The LDL-C percentiles values are in mg/dL, represented by black (e_COR) and red (PHC) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for LDL-C is 0 to 250. Scale for percentiles is 0 to 1.0.

7.3.6. Supplementary Figure 6



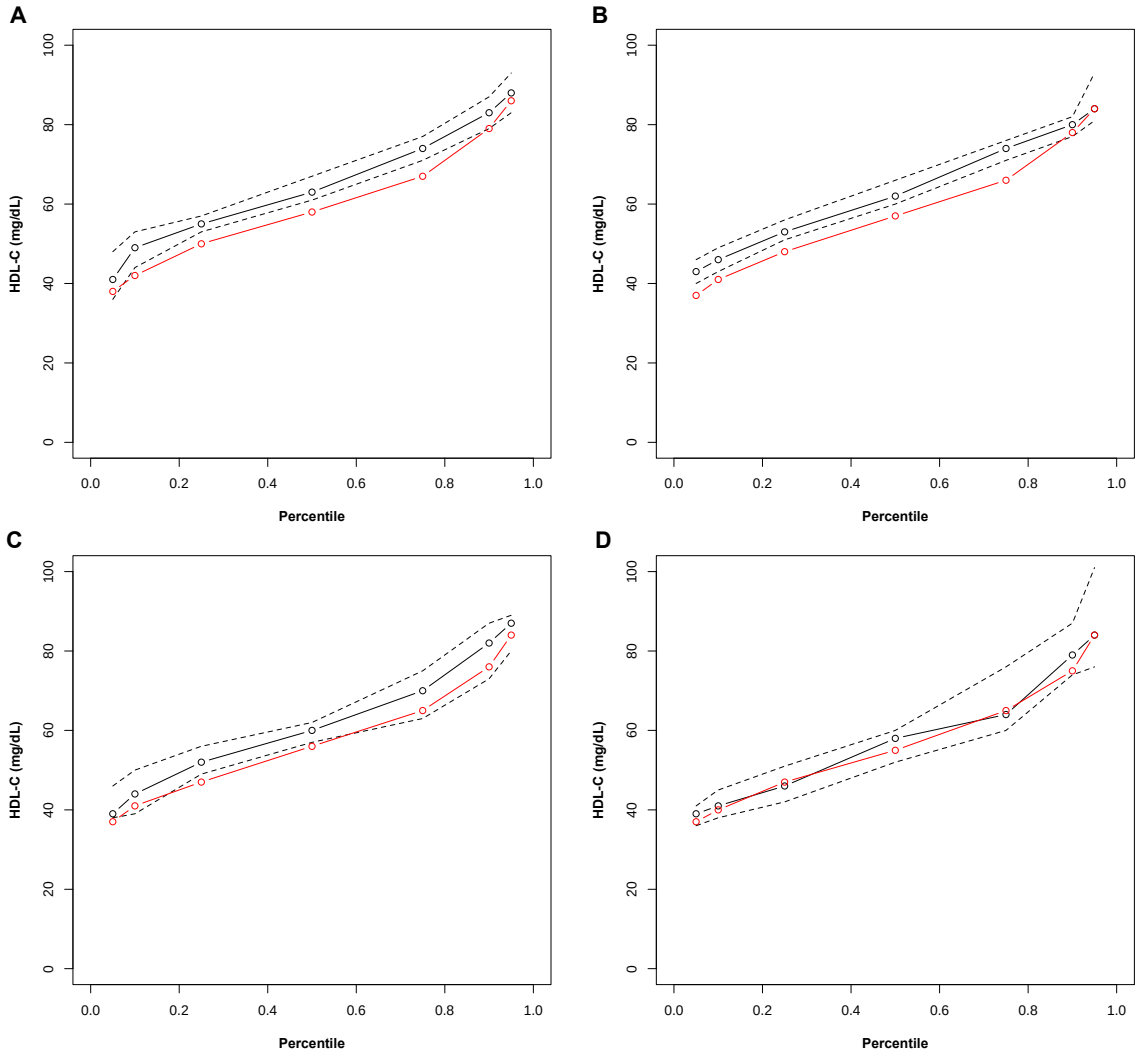
Supplementary Figure 6 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the LDL-C in women, between e_COR Study and the Primary health care (PHC) users Study in Portugal, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the primary health care (PHC) users in Portugal in 2013 in women age groups 18-29 (**A**), 30-39 (**B**), 40-49 (**C**), and 50-59 (**D**). The LDL-C percentiles values are in mg/dL, represented by black (e_COR) and red (PHC) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for LDL-C is 0 to 250. Scale for percentiles is 0 to 1.0.

7.3.7. Supplementary Figure 7



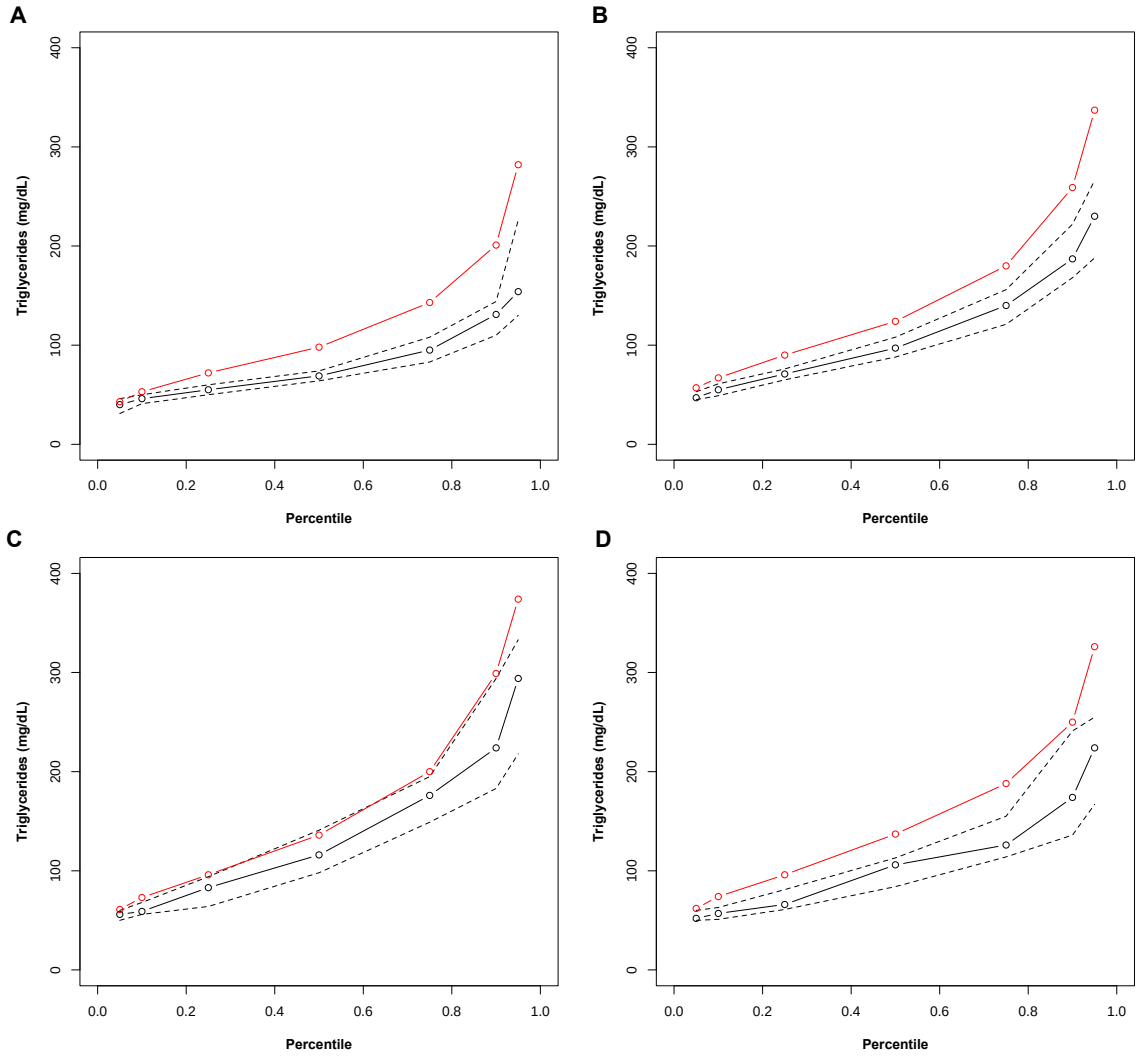
Supplementary Figure 7 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the HDL-C in men, between e_COR Study and the Primary health care (PHC) users Study in Portugal, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the primary health care (PHC) users in Portugal in 2013 in men age groups 18-29 (**A**), 30-39 (**B**), 40-49 (**C**), and 50-59 (**D**). The HDL-C percentiles values are in mg/dL, represented by black (e_COR) and red (PHC) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for HDL-C is 0 to 100. Scale for percentiles is 0 to 1.0.

7.3.8. Supplementary Figure 8



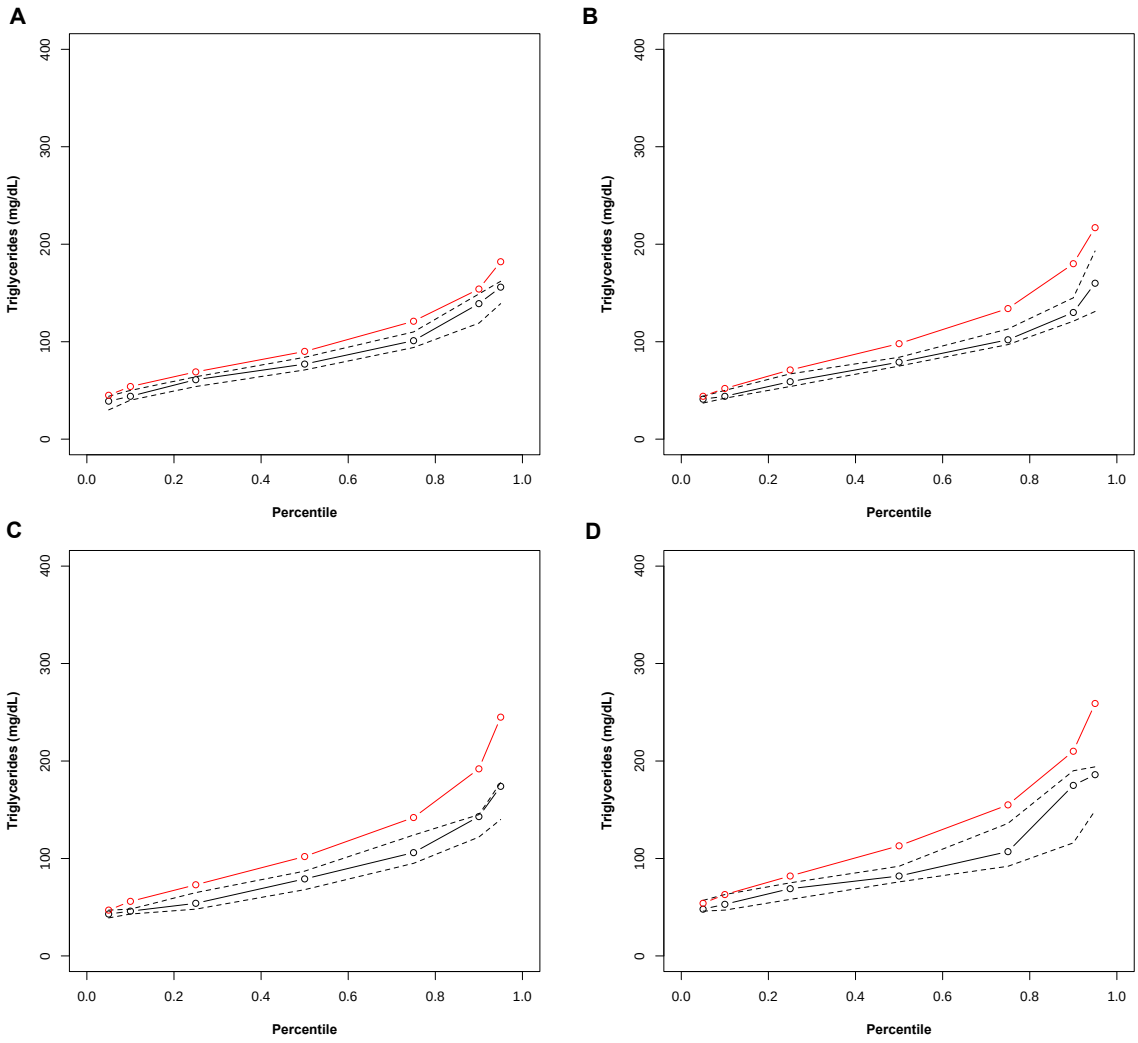
Supplementary Figure 8 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the HDL-C in women, between e_COR Study and the Primary health care (PHC) users Study in Portugal, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the primary health care (PHC) users in Portugal in 2013 in women age groups 18-29 (A), 30-39 (B), 40-49 (C), and 50-59 (D). The HDL-C percentiles values are in mg/dL, represented by black (e_COR) and red (PHC) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for HDL-C is 0 to 100. Scale for percentiles is 0 to 1.0.

7.3.9. Supplementary Figure 9



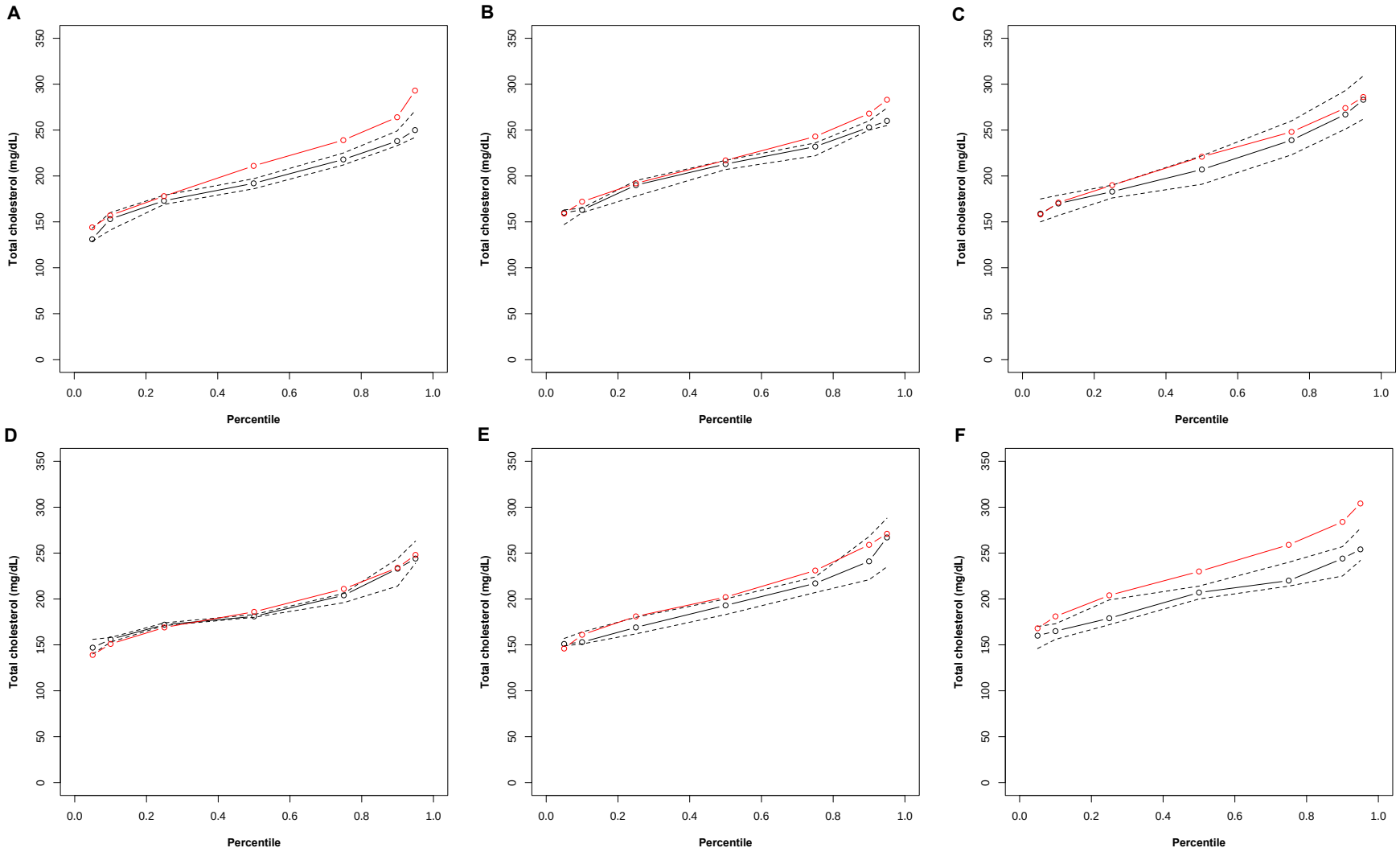
Supplementary Figure 9 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the triglycerides (TG) in men, between e_COR Study and the Primary health care (PHC) users Study in Portugal, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the primary health care (PHC) users in Portugal in 2013 in men age groups 18-29 (**A**), 30-39 (**B**), 40-49 (**C**), and 50-59 (**D**). The TG percentiles values are in mg/dL, represented by black (e_COR) and red (PHC) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for TG is 0 to 400. Scale for percentiles is 0 to 1.0.

7.3.10. Supplementary Figure 10



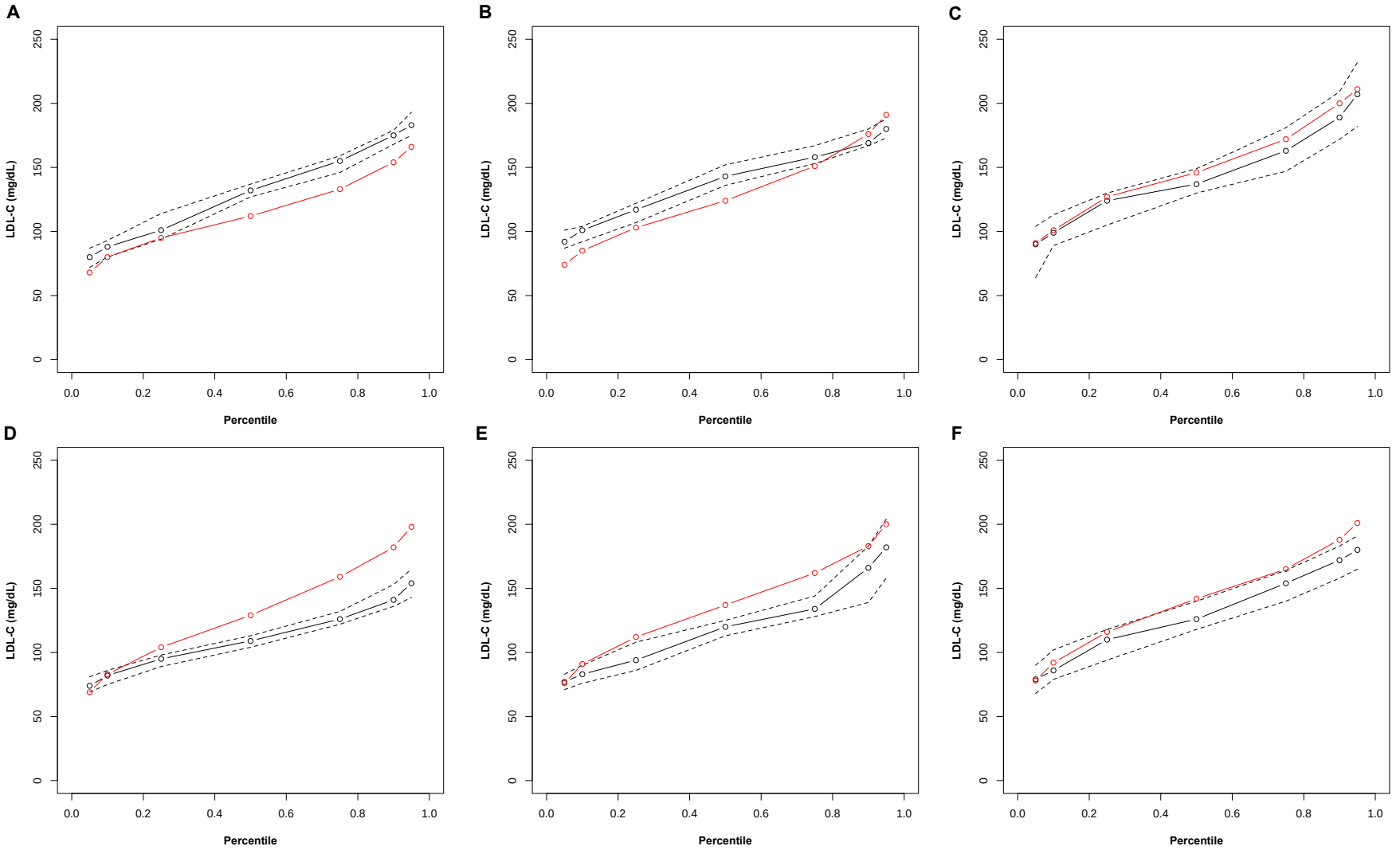
Supplementary Figure 10 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the triglycerides (TG) in women, between e_COR Study and the Primary health care (PHC) users Study in Portugal, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the primary health care (PHC) users in Portugal in 2013 in women age groups 18-29 (**A**), 30-39 (B), 40-49 (**C**), and 50-59 (**D**). The TG percentiles values are in mg/dL, represented by black (e_COR) and red (PHC) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for TG is 0 to 400. Scale for percentiles is 0 to 1.0.

7.3.11. Supplementary Figure 11



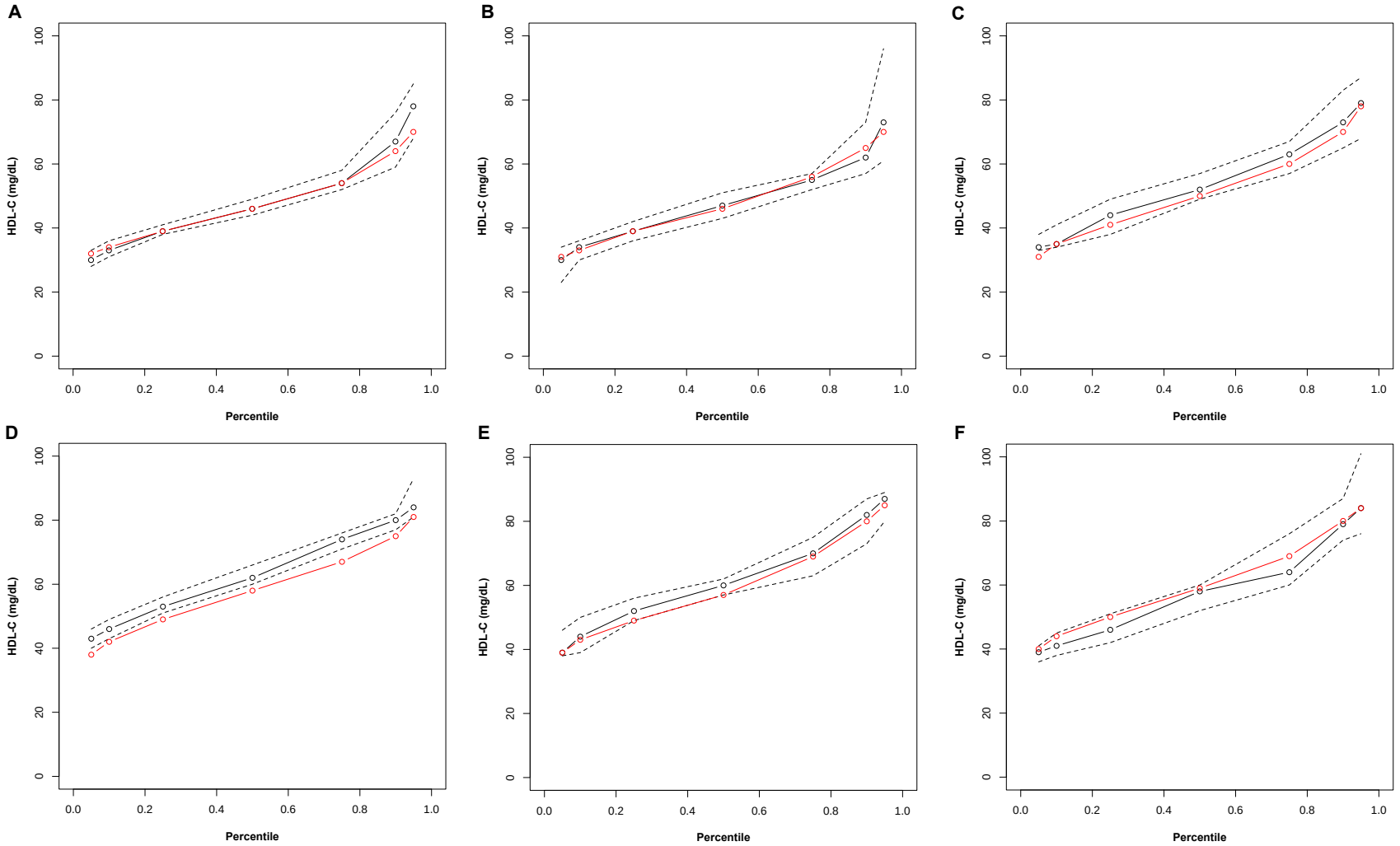
Supplementary Figure 11 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the total cholesterol (TC) in men and women, between e_COR Study in Portugal and the DRECE Study in Spain, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the Spanish population (DRECE Study) in men age groups 30-39 (**A**), 40-49 (**B**), and 50-59 (**C**), and for women age groups 30-39 (**D**), 40-49 (**E**), and 50-59 (**F**). The TC percentiles values are in mg/dL, represented by black (e_COR) and red (DRECE) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for TC is 0 to 350. Scale for percentiles is 0 to 1.0.

7.3.12. Supplementary Figure 12



Supplementary Figure 12 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the LDL-C in men and women, between e_COR Study in Portugal and the DRECE Study in Spain, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the Spanish population (DRECE Study) in men age groups 30-39 (**A**), 40-49 (**B**), and 50-59 (**C**), and for women age groups 30-39 (**D**), 40-49 (**E**), and 50-59 (**F**). The LDL-C percentiles values are in mg/dL, represented by black (e_COR) and red (DRECE) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for LDL-C is 0 to 250. Scale for percentiles is 0 to 1.0.

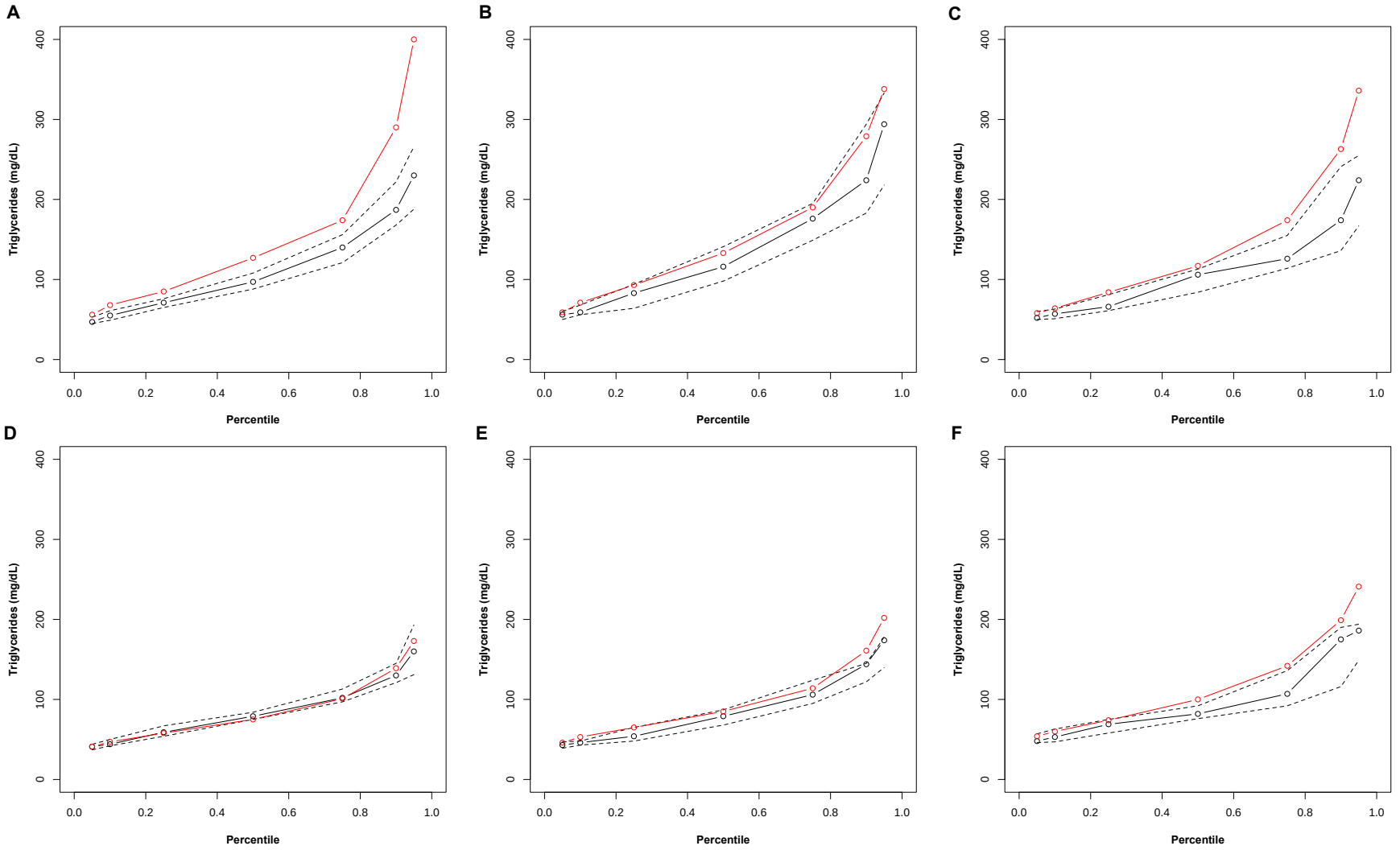
7.3.13. Supplementary Figure 13



Supplementary Figure 13 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the HDL-C in men and women, between e_COR Study in Portugal and the DRECE Study in Spain, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles

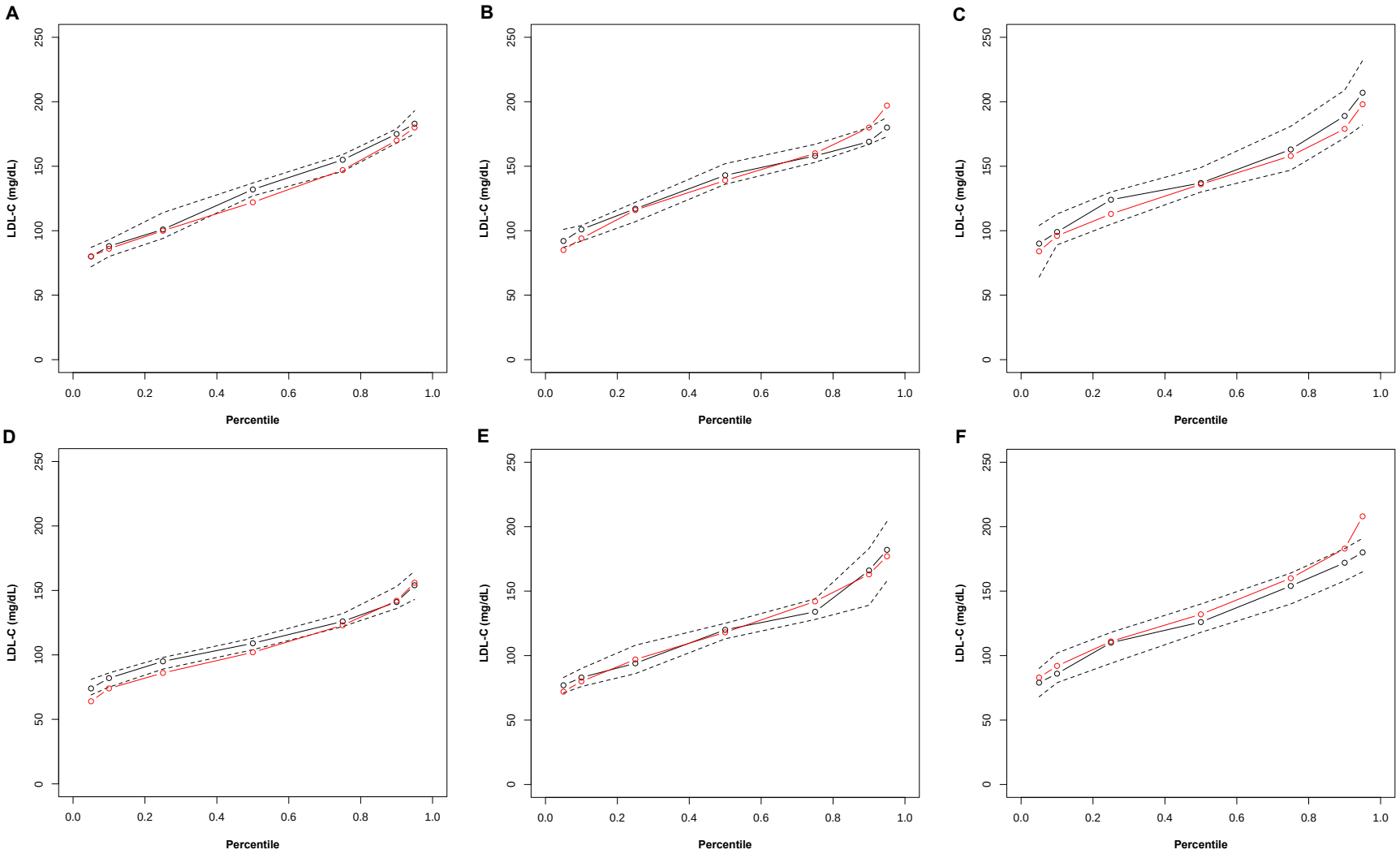
estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the Spanish population (DRECE Study) in men age groups 30-39 (**A**), 40-49 (**B**), and 50-59 (**C**), and for women age groups 30-39 (**D**), 40-49 (**E**), and 50-59 (**F**). The LDL-C percentiles values are in mg/dL, represented by black (e_COR) and red (DRECE) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for HDL-C is 0 to 100. Scale for percentiles is 0 to 1.0.

7.3.14. Supplementary Figure 14



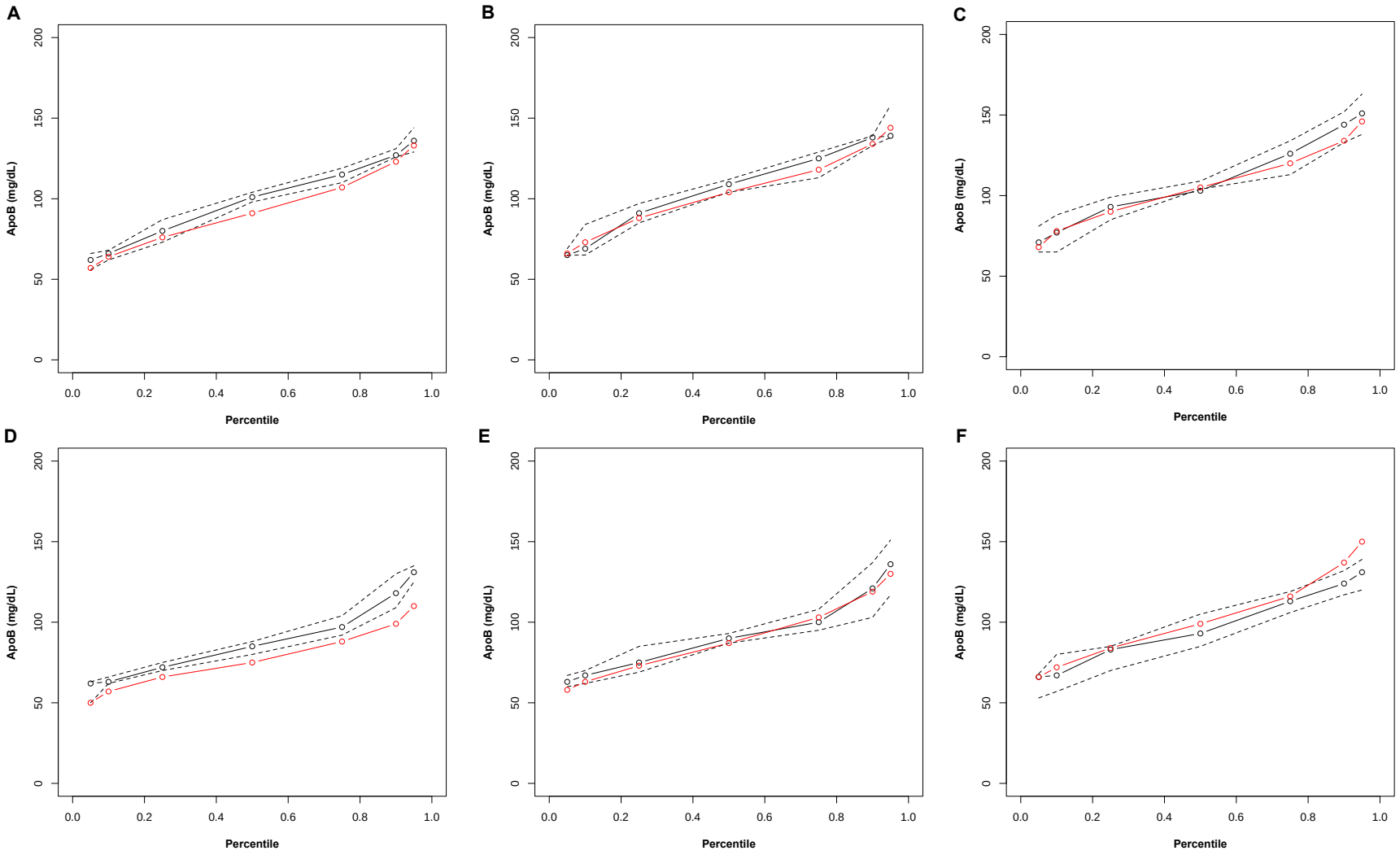
Supplementary Figure 14 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the triglycerides (TG) in men and women, between e_COR Study in Portugal and the DRECE Study in Spain, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the Spanish population (DRECE Study) in men age groups 30-39 (**A**), 40-49 (**B**), and 50-59 (**C**), and for women age groups 30-39 (**D**), 40-49 (**E**), and 50-59 (**F**). The TG percentiles values are in mg/dL, represented by black (e_COR) and red (DRECE) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for TG is 0 to 400. Scale for percentiles is 0 to 1.0.

7.3.15. Supplementary Figure 15



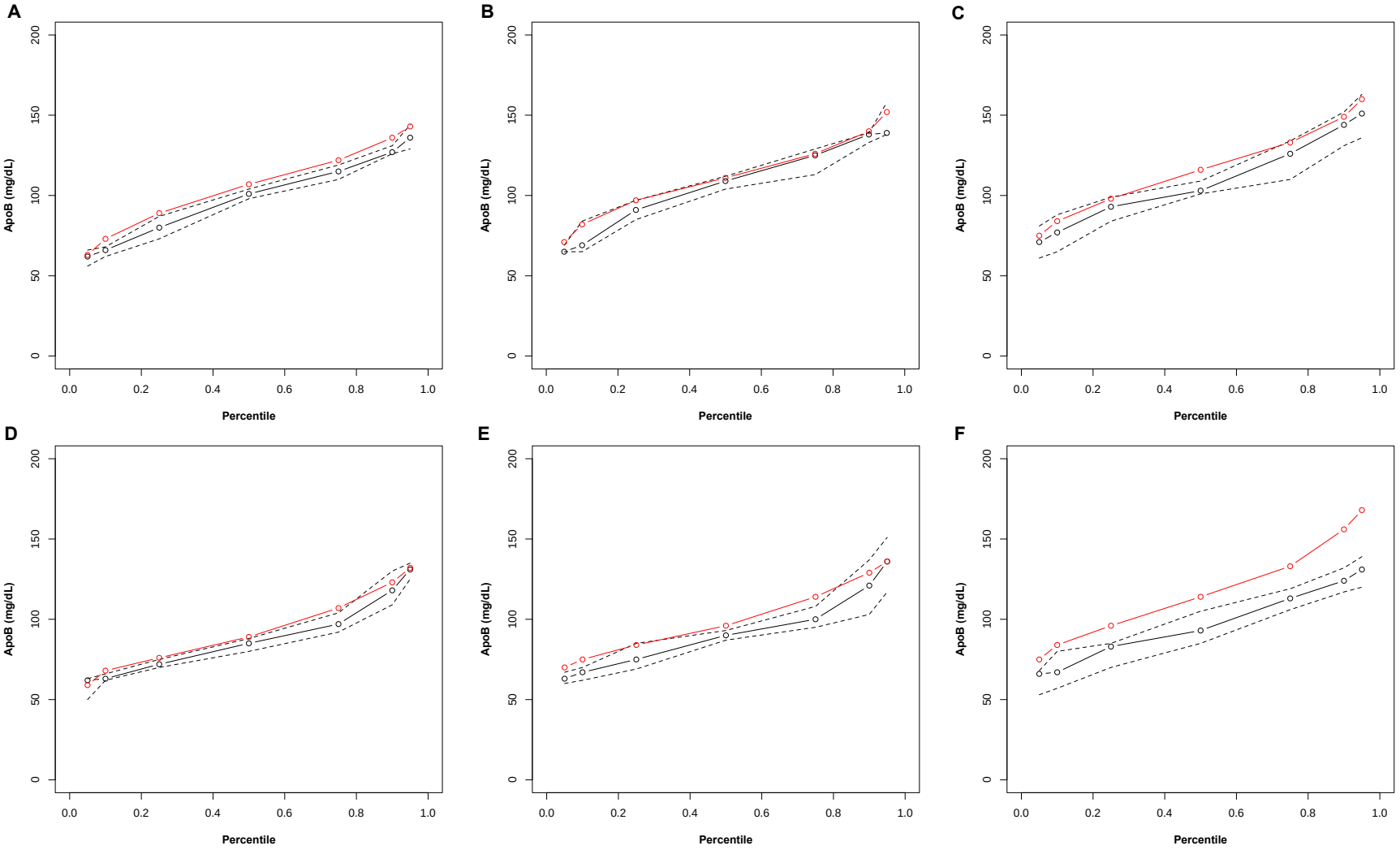
Supplementary Figure 15 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the LDL-C in men and women, between e_COR Study in Portugal and the Framingham Offspring Study in the United States of America, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the American population (Framingham Offspring Study) in men age groups 30-39 (**A**), 40-49 (**B**), and 50-59 (**C**), and for women age groups 30-39 (**D**), 40-49 (**E**), and 50-59 (**F**). The LDL-C percentiles values are in mg/dL, represented by black (e_COR) and red (Framingham Offspring) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted line. Scale for LDL-C is 0 to 250. Scale for percentiles is 0 to 1.0.

7.3.16. Supplementary Figure 16



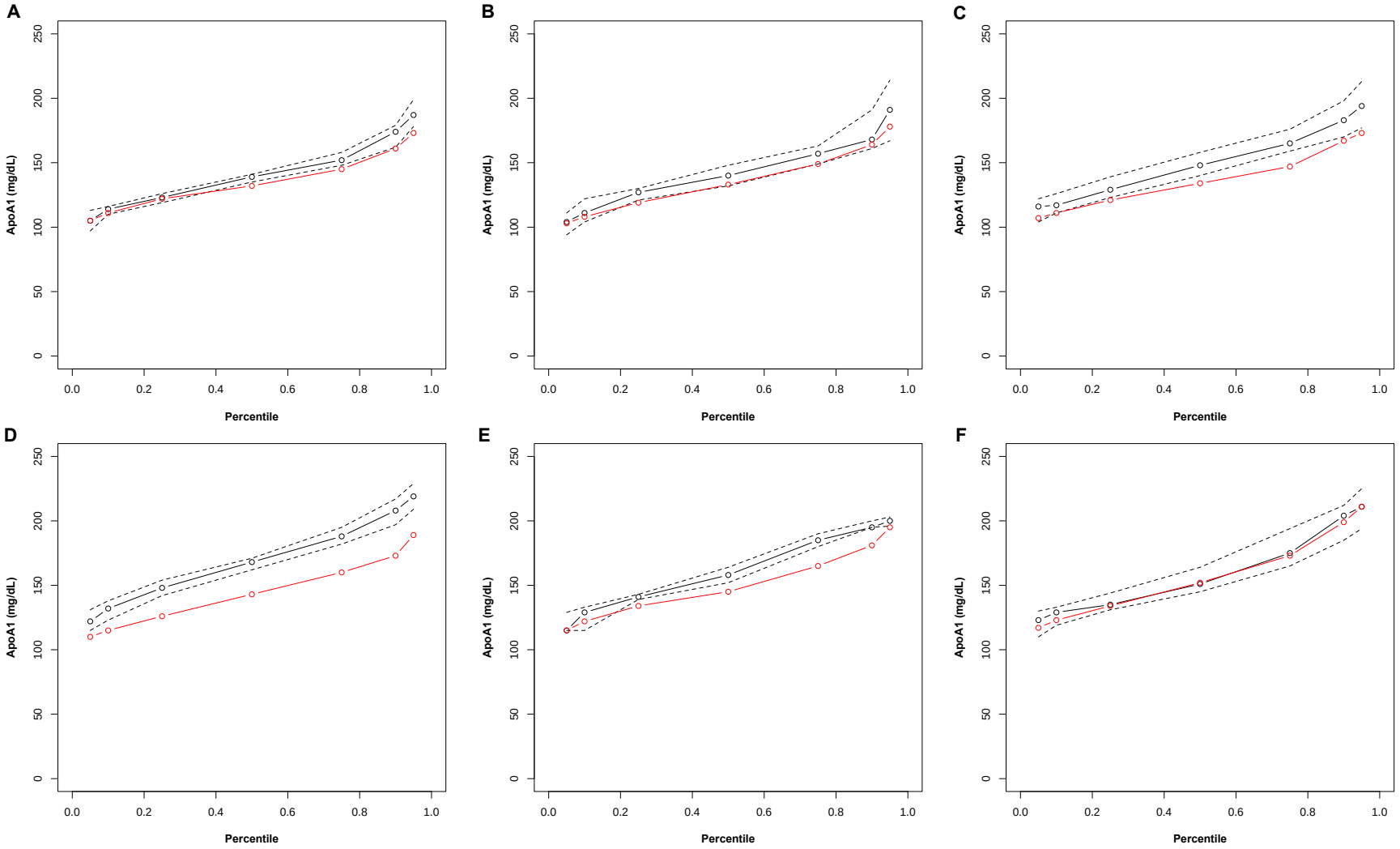
Supplementary Figure 16 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the apolipoprotein B (apoB) in men and women, between e_COR Study in Portugal and the Framingham Offspring Study in the United States of America, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the American population (Framingham Offspring Study) in men age groups 30-39 (**A**), 40-49 (**B**), and 50-59 (**C**), and for women age groups 30-39 (**D**), 40-49 (**E**), and 50-59 (**F**). The LDL-C percentiles values are in mg/dL, represented by black (e_COR) and red (Framingham Offspring) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for apoB is 0 to 200. Scale for percentiles is 0 to 1.0.

7.3.17. Supplementary Figure 17



Supplementary Figure 17 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the apolipoprotein B (apoB) in men and women, between e_COR Study in Portugal and the National Health and Nutrition Examination Survey III (NHANES III) in the United States of America, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the American population (NHANES III) in men age groups 30-39 (**A**), 40-49 (**B**), and 50-59 (**C**), and for women age groups 30-39 (**D**), 40-49 (**E**), and 50-59 (**F**). The LDL-C percentiles values are in mg/dL, represented by black (e_COR) and red (NHANES III) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for apoB is 0 to 200. Scale for percentiles is 0 to 1.0.

7.3.18. Supplementary Figure 18



Supplementary Figure 18 – Comparison of the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles of the apolipoprotein A1 (apoA1) in men and women, between e_COR Study in Portugal and the National Health and Nutrition Examination Survey III (NHANES III) in the United States of America, using dot plot graphs constructed with the 95% CI estimated for the e_COR Study percentiles. The percentiles estimated for the Portuguese population in the present study (e_COR) were compared with the percentiles estimated for the American population (NHANES III) in men age groups 30-39 (**A**), 40-49 (**B**), and 50-59 (**C**), and for women age groups 30-39 (**D**), 40-49 (**E**), and 50-59 (**F**). The LDL-C percentiles values are in mg/dL, represented by black (e_COR) and red (NHANES III) solid lines. The 95% CI for e_COR Study percentiles values are represented by black dotted lines. Scale for apoA1 is 0 to 250. Scale for percentiles is 0 to 1.0.

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Chapter 3

e_LIPID – BIOCHEMICAL AND GENETIC CHARACTERISATION OF THE PORTUGUESE POPULATION: CONSIDERATIONS FOR LIPID METABOLISM ASSESSMENT

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ABSTRACT

The incidence of cardiovascular diseases (CVD) has been increasing in low and middle-income countries as a result of the modification of lifestyles and increased prevalence of cardiovascular risk factors. Since dyslipidaemia is one of the major independent cardiovascular risk factors, their correct identification is of great importance to implement specific interventions for CVD prevention. The aim of the present study was to characterise the lipid profile of the Portuguese population by a biochemical and genetic approach. Overall, 1,688 individuals from the Portuguese population (e_COR Study) were included. Population specific percentiles for ten lipid and lipoprotein biomarkers were used as reference values to characterise the dyslipidaemia. For association between lipids and non-lipid/other cardiovascular risk factors, Pearson correlation was used. Genetic studies were performed by Sanger and next generation sequencing. A high prevalence of severe dyslipidaemia (>90th percentile) was found being the highest values found for total cholesterol, apolipoprotein B (apoB), and low-density lipoprotein cholesterol (LDL-C) (16.22%, 16.02%, and 15.85%, respectively). The use of population specific values for age and sex revealed that dyslipidaemia is under-diagnosed in women. Correlation between apoB and small, dense LDL-C (sdLDL-C) with other lipid and non-lipid risk factors was found adding evidence for the importance of apoB and sdLDL-C determination. Additionally, three individuals were found have a functional mutation causing familial hypercholesterolaemia. For dyslipidaemia assessment in a population it is important to use population age and sex specific values. Although high values of dyslipidaemia have been found, dyslipidaemia is a modifiable cardiovascular risk factor that can be tackled by life styles modifications and a more personalized treatment. However changes in health policies must be made.

KEYWORDS

Cardiovascular risk factors; Dyslipidaemia; Lipid profile; Lipids percentiles; at risk values; Atherogenic risk; Apolipoprotein B; Small, dense low-density lipoprotein cholesterol.

1.INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death among non-communicable diseases (NCDs) and disabilities worldwide, both in developed and developing countries, accounting for 47% of deaths in Europe (Nichols et al., 2012; GBD 2013 Mortality and Causes of Death Collaborators et al., 2015). CVD has a multifactorial aetiology with a number of potentially modifiable risk factors. Dyslipidaemia, hypertension (HT) and cigarette smoking are three well-known major yet modifiable risk factors for CVD. Control of major cardiovascular risk factors has been revealed to definitively decrease the risk of CVD (Gielen and Landmesser, 2014).

Epidemiological studies have linked CVD to increasing values in plasma lipids, such as total cholesterol (TC), non-high-density lipoprotein cholesterol (non-HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) and also to low concentration of high-density lipoprotein cholesterol (HDL-C); alone and together, these changes contribute to the development of atherosclerosis (Perk et al., 2012).

The central role of dyslipidaemia as a major contributor to CVD risk has been highlighted by the global case-control INTERHEART study, in which the apolipoprotein B100 (apoB)/apolipoprotein A1 (apoA1) ratio had the highest population-attributable risk (54%) and the highest odds ratio (OR) with each 1 standard deviation (SD) difference [1.59; 95% confidence interval (1.53–1.64)]. The apoB/apoA1 ratio was superior to any of the cholesterol ratios for estimation of the risk of acute myocardial infarction in all ethnic groups, in both sexes, and at all ages (Yusuf et al., 2004b). The 2002 World Health Report indicated that high plasma TC levels are responsible for 56% of CHD and 18% of nonfatal cerebrovascular diseases worldwide (Guilbert, 2003).

Extensive evidence from large-scale prospective studies has demonstrated that LDL-C lowering therapies (primarily statins) substantially reduce risk of CVD events in patients at high risk of any type of major vascular event: for every 1 mmol/L (39 mg/dL) decrease in LDL-C, the risk of major cardiovascular events is decreased by 21% (Bonovas et al., 2011).

The impact of the global epidemics of metabolic syndrome, obesity and type 2 diabetes mellitus carry a high proportion of patients with complex lipid abnormalities, which are not restricted to elevated LDL-C or TC levels but often comprise reduced levels of HDL-C, and/or elevated TG, non-HDL-C and small dense LDL (sdLDL) (Sardinha et al., 2012).

Several studies demonstrated that sdLDL particles co-exists with other atherogenic risk factors like high TG and apoB, and low HDL-C, and was also identified as the best

predictor of CVD, being referred as a very important risk factor for atherosclerosis (Austin et al., 1988; Gardner et al., 1996).

The prevalence of dyslipidaemia can vary across population groups according to nationality, ethnicity, genetics and socio-cultural and economic factors. Because the population is aging, a periodic assessment of the prevalence of cardiovascular risk factors is necessary. This information will help to predict cardiovascular mortality trends for the following years and to design preventive strategies to cope with this health problem.

In 2013, Cortez-Dias and colleagues also characterised the dyslipidaemia patterns in their sample but does not correspond to the general population (Cortez-Dias et al., 2013). So the last assessment of the dyslipidaemia patterns in Portugal was performed in 2001 in a project funded by Becel (Instituto de Alimentação Becel, 2002); no other population study has been done for dyslipidaemia since that time.

The aim of the present study was to characterise the lipid profile and the distribution of TC, LDL-C, HDL-C, TG, apoA1, apoB, non-HDL-C, small, dense LDL cholesterol (sdLDL-C), to estimate the prevalence of dyslipidaemia in Portugal, by using reference values based on percentiles for lipid metabolism biomarkers previously determined for the Portuguese population, and finally to analyse the association among lipid and non-lipid/other cardiovascular risk factors. To the best of our knowledge, this is the most complete characterisation of the lipid profile of the Portuguese population with discrimination of results for sdLDL-C not commonly seen in any worldwide lipid characterisation.

2. MATERIALS AND METHODS

2.1. Study population

This study included initially 1,688 unrelated adults (98% Caucasians), 848 men and 840 women aged between 18 and 79 years, from Norte, Centro, Lisboa, Alentejo, and Algarve regions of Portugal. All samples and demographic and clinical data used in the present study were obtained from e_COR Study, a pre-designed and developed cross-sectional epidemiological study performed by our research group with the major aimed to determine the prevalence of cardiovascular risk factors of the Portuguese population (Bourbon et al., 2018). The present project was developed to perform an extensive characterisation of one of the major cardiovascular risk factors, dyslipidaemia. The e_COR Study was approved by the National Data Collection Commission and National Institute of Health (INSA) Ethic Committee, and participants gave informed consent to each aspect of the study.

2.2. Data collection

Demographic and clinical data on cardiovascular risk factors were obtained in the scope of the e_COR Study (Bourbon et al., 2018). All data was obtained between January 2012 and December 2014, by specialized laboratory technicians and/or nurses. A resume of the e_COR methods is presented in Supplementary data.

2.3. Biochemical analysis

For each individual, 12 hours fasting blood samples were collected. Biochemical analysis was performed in a central laboratory and methods are described in Supplementary data.

Non-HDL-C values was calculated as previously described (Catapano et al., 2016; Nordestgaard et al., 2016): TC minus HDL-C.

2.4. Dyslipidaemia characterisation

2.4.1. Characterisation of the lipid profile

For this analysis the whole e_COR population was included (1,688 adults, 848 men and 840 women aged between 18 and 79) involving Norte, Centro, Lisboa, Alentejo and Algarve regions. The e_COR sample was stratified by region, but not representative of the Portuguese population, so stratified random sampling techniques were applied based on real population (Cochran, 1977). These techniques allowed us to build a weighed

estimator of the mean values and their correspondent 95% CI, standard deviation (SD), variance, and standard error (SE), as well as of the prevalence (expressed as percentage) and their correspondent 95% CI, with known asymptotic probabilistic behaviour leading to the calculation of these estimations. For such estimation, population was divided into non-overlapping subpopulations called strata, comprising the whole of the population (Figure 1). For that, stratum weights were calculated in each region, Norte, Centro, Lisboa, Alentejo and Algarve, by gender and age, according to the demographic composition of the adult population resident in Portugal in 2011(Instituto Nacional de Estatistica, Censos 2011). Cases with secondary causes of dyslipidaemia, such as diabetes, hyperthyroidism and hypothyroidism were excluded for the estimation of the number of individuals in our sample presenting criteria for possible monogenic causes of dyslipidaemia.

As reference values for the analysis of the prevalence of dyslipidaemia, we used the percentiles values previously estimated for the adult Portuguese population (Bourbon et al., *Submitted*). The 25^{th}-75^{th} percentile was considered within recommended, above the 90^{th} percentile ($P90^{th}$) for TC, LDL-C, TG, apoB, non-HDL-C, and sdLDL-C or below the 10^{th} percentile ($P10^{th}$) for HDL-C and apoA1 was considered risk, and so it was defined as the cut off for each lipid disorder. High risk was defined above the 95^{th} ($P95^{th}$) or below the 5^{th} ($P5^{th}$) percentiles. The high risk for apoB/apoA1 and sdLDL-C/LDL-C ratios was defined above the 90^{th} ($P90^{th}$). In cases where sample size was small, such as monogenic dyslipidaemia, or the number of individuals to be above the $P90^{th}$ prior to medication who reached values below the $P50^{th}$ for TC, LDL-C and for apoB, we have only mentioned the number of individuals in our sample that presented the characteristics of interest (and not the estimation of the prevalence) expressed as percentage.

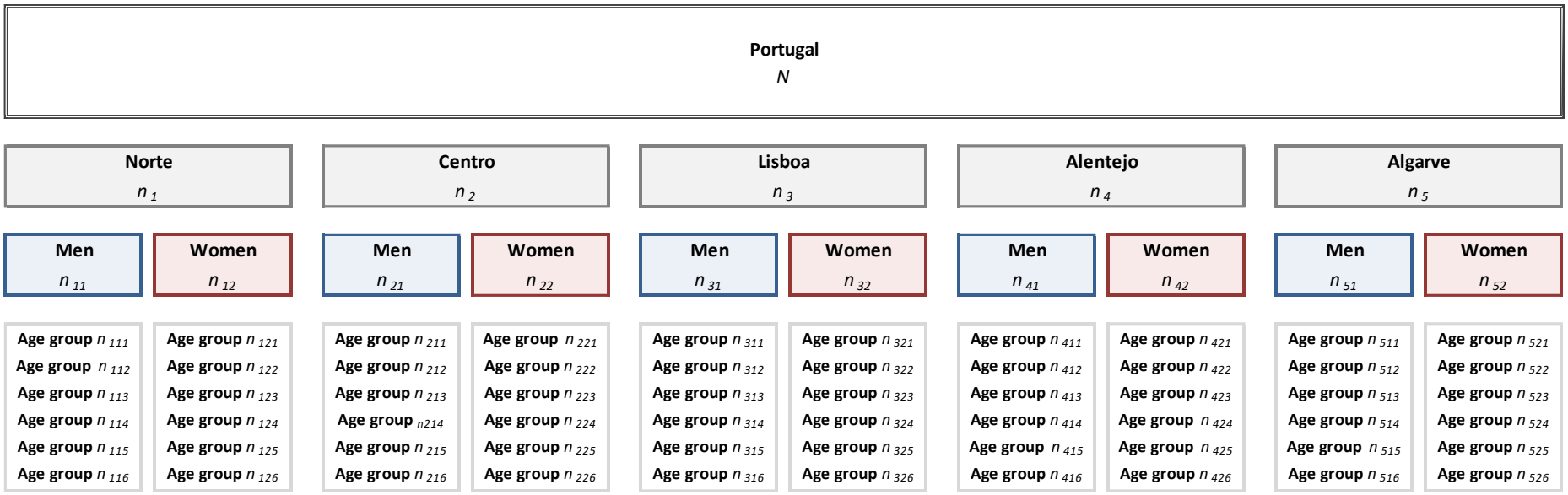

Figure 1 – Schematic representation of sample stratum. Population sample is composed of N units divided into subpopulations of n units that together comprise the whole of the population (N). Estimation of the mean values, variances and proportions should be obtained by a different combination of the strata, changing the hierarchy represented in the diagram.

2.4.2. Corrections factors regarding lipid-lowering therapy

For dyslipidaemia characterisation, we accounted for lipid-lowering therapy in medicated participants (with statins) so published correction factors were used to estimate untreated values for TC, LDL-C (Baigent et al., 2005; Sniderman, 2008; Peloso et al., 2014; Khera et al., 2016) and apoB (Sniderman, 2008): measured TC, LDL-C and apoB was divided by 0.8 (20% TC reduction), 0.7 (30% LDL-C reduction) and 0.763 (23.7% apoB reduction, corresponding to 79% of the LDL-C reduction), respectively. Untreated TG, HDL-C, apoA1 and sdLDL-C values were not estimated, since the effects of lipid-lowering therapy with statins apparently are not significant in these biomarkers, and there is controversial evidences of its benefit on sdLDL-C (Tilly-Kiesi, 1991; März et al., 2001; Baldassarre et al., 2005; Barter et al., 2006; Tokuno et al., 2007; Sniderman, 2008; Florentin et al., 2011; Yoshino et al., 2012; Diffenderfer and Schaefer, 2014; Nishikido et al., 2016). Only a very small number of participants was under medication to lower TG, so corrections factors in these cases were not considered.

For the apoB/apoA1 and sdLDL-C/LDL-C ratios analysis, we used the apoB or LDL-C values without correction factors in medicated participants, since the objective was to evaluate the present atherogenic risk of the Portuguese population.

2.4.3. Non-lipid parameters

Variables for non-lipid parameters were classified according European guidelines and World Health Organization recommendations as previously described in e_COR Study (Bourbon et al., 2018) (Supplementary data).

2.4.4. General statistical analysis

The collected data were weighted by gender, age and geographic region (NUTS II) based on CENSUS 2011(Instituto Nacional de Estatistica, Censos 2011), to obtain representative results of the mainland Portuguese population.

Statistical analyses were performed using R (version 3.1.2) software (R: The R Project for Statistical Computing). Statistical models were developed to characterise the lipid profile of the Portuguese population and identify existing associations between lipids and apolipoproteins and other cardiovascular risk factors.

For comparison analysis of the lipids and lipoproteins plasma concentration values between independent groups, non-parametric tests were applied using Kruskal-Wallis test for more than two independent samples, or Two-sample Wilcoxon test for two independent samples. When there were assumptions of normality (Shapiro-Wilk or

Kolmogorov-Smirnov tests) and homogeneity of variance (Bartlett test), parametric tests were applied using ANOVA test for more than two independent samples or Student t test for two independent samples. For comparison analysis of the prevalence, the 95% confidence intervals (CI) were used. Whenever the two CI non-overlap, we could immediately conclude that there was evidence to conclude that the proportions are statistically different. In the remaining cases (overlap of the two proportions confidence intervals), the two proportions were compared using the Z-value. For the calculation of the test statistic, the weighted estimates of the proportions and correspondent variances were used. The hypothesis of equality of two proportions was tested against the alternative that they are not equal. P value < 0.05 was considered significant.

2.4.5. Correlations among lipid and non-lipid risk factors

Pearson correlation was used to evaluate the association between dependent variables, like lipids biochemical biomarkers, and independent variables, namely age, body mass index (BMI), alcohol intake, systolic (SBP) and diastolic (DBP) blood pressure, and glucose.

2.4.6. Logistic regression analyses to identify relationship between dyslipidaemia and other cardiovascular risk factors

Binary logistic regression was used to obtain odds ratios (ORs) and their 95% CI for dyslipidaemia considering other cardiovascular risk factors, namely prehypertension, HT, alcohol intake, overweight and obesity. For this analysis, values of TC, LDL-C, apoB, TG, and sdLDL-C above the 90th percentile, as well as HDL-C and apoA1 below the 10th percentile were considered as dyslipidaemia. The estimation of ORs was adjusted by gender, age, lipid-lowering therapy and hormone users (women), and for medical history of diabetes, hyperthyroidism, and hypothyroidism. P value < 0.05 was considered as significant.

2.4.7. Possible monogenic cause of dyslipidaemias

The criterion of “possible Familial Hypercholesterolaemia (FH)” was applied according to the Simon Broome Heart Research Trust (1991) (Simon Broome Register Group, 1991) (Supplementary data). To search individuals in the very extreme percentiles, the 1th (P1th) and 99th (P99th) percentiles were estimated for this purpose, as previously reported (Bourbon et al., *Submitted*). Cases with secondary causes of dyslipidaemia, such as diabetes, hyperthyroidism and hypothyroidism were excluded for the estimation of

the number of individuals in our sample presenting criteria for possible monogenic causes of dyslipidaemia.

2.5. Molecular analysis

2.5.1. Monogenic Familial Hypercholesterolaemia by Sanger sequencing

From a total of 62 individuals from the e_COR Study with LDL-C or TC above the P95th, and with family history of premature CVD (pCVD) or hypercholesterolaemia, we selected 33 individuals with Simon Broome clinical criteria for FH to perform the genetic diagnosis. The genetic diagnosis of FH was performed by the molecular analysis of *APOB* (two fragments of exons 26 and 29), *LDLR* (including the study of splice regions and large rearrangements), and *PCSK9* genes, as previously reported (Medeiros et al., 2010).

2.5.2. Other causes for monogenic dyslipidaemia by targeted sequencing in the extremes phenotypes

A total of 29 individuals from e_COR Study were selected for further studies by next-generation sequencing with a targeted panel of 26 genes associated with dyslipidaemia due to: 1) be in the extreme low percentile (P1th) for LDL-C and/or apoB (n=7); 2) be in the P1th for HDL-C (n=5); 3) be in the extreme high percentile (P99th) for HDL-C (n=5); 4) be in P99th for TG (n=12) (Supplementary Table 5).

Targeted sequencing included all exons and untranslated regions (UTRs) of all 26 genes. The NGS libraries were prepared using the SureSelect^{QXT} Target Enrichment for Illumina Multiplex Sequencing (Agilent Technologies, 2016) (Agilent Technologies, Santa Clara, CA, USA) as described in Supplementary data. Of targeted regions, 97% were covered at ≥ Q30. The generated FASTQ files were aligned to the human genome reference GRCh37 (hg19) and scrutinized using SureCall data analysis software (version 3.0; Agilent Technologies, Santa Clara, CA, USA) (Agilent Technologies, 2015). The median read depth was 239.00 (IQR 111-303) (ranging from 10 to 951).

Data analysis workflow regarding the 26 genes are described in Supplementary Figure 1.

3. RESULTS

3.1. Characterisation of the lipid profile

3.1.2. Evaluation of dyslipidaemia by percentiles

3.1.2.1. Analysis of the prevalence of dyslipidaemia (90th or 10th percentiles)

All 1,688 individuals (Supplementary Table 1) were considered for the characterisation of dyslipidaemia in the Portuguese population (except for the apoB (N=1,687) and sdLDL-C (N=1,667)). For individuals aged between 60 and 79 years, the percentiles of the age group 50-59 were used as reference (Bourbon et al., *Submitted*). Stratified random sampling techniques were used for the prevalence estimation. Main results are presented in Table 1. Results showed that the estimated prevalence in the Portuguese population of hypercholesterolaemia for TC and LDL-C, above the P90th, was 16.22% and 15.85%, respectively. When analysed by gender for TC above the P90th, the prevalence was 12.40% and 19.68% for men and women, respectively; for LDL-C above the P90th, the prevalence was 12.90% and 18.53% for men and women, respectively. Low levels of HDL-C (HDL-C below the P10th) and high levels of triglycerides (TG above the P90th) were less prevalent, affecting 9.73% (9.21% of men and 10.20% of women), and 13.63% (15.93% in men and 11.54% in women), respectively. Considering the apolipoproteins, the prevalence for low levels of apoA1 concentration (apoA1 below the P10th) was 10.98% (11.36% of men and 10.45% of women), and the prevalence for high concentrations of apoB (apoB above the P90th) was 16.02% (13.07% for men and 18.70% for women). For the high concentration levels of non-HDL-C and sdLDL-C (above the P90th), the prevalence was 15.46% and 10.59%, respectively. When analysed by gender, the prevalence for non-HDL-C above the P90th was 11.69% for men and18.89% for women, and for sdLDL-C above the P90th was 9.42% for men and 11.64% for women. The highest prevalence of individuals above the P90th for TC, LDL-C, apoB, non-HDL-C and sdLDL-C in the Portuguese population were observed in women, while hypertriglyceridaemia and low levels of HDL-C and apoA1 were more prevalent in men. Being women is a protection factor for the risk of developing hypertriglyceridaemia or having low levels of HDL-C (OR=0.36 CI=[0.22- 0.58] (P<0.001), and OR=0.52 CI=[0.29-0.92] (P<0.05) for hypertriglyceridaemia and low HDL-C, respectively); in contrast to men (OR=2.00 CI=[1.44-2.79] (P<0.001) and OR=2.18 CI=[1.56-3.05] (P<0.001) for hypertriglyceridaemia and low HDL-C, respectively).

Table 1 – Estimated prevalence of individuals above the 90th or below the 10th percentiles.

		Prevalence of individuals above the 90th or below the 10th percentiles												
		Total				Men				Women				
Lipid biomarker	**Percentile**	**n**	%	95% CI		**n**	%	95% CI		**n**	%	95% CI		**P value[a]**
Total cholesterol	>90th	276	16.22	14.45	17.98	111	12.40	10.02	14.79	165	19.68	16.90	22.45	<0.001
LDL-C	>90th	264	15.85	14.10	17.60	109	12.90	10.44	15.36	155	18.53	15.80	21.25	<0.001
HDL-C	<10th	154	9.72	8.30	11.14	74	9.26	7.19	11.33	80	10.13	7.96	12.30	0.274
Triglycerides	>90th	215	13.63	11.99	15.26	126	15.93	13.30	18.55	89	11.54	9.25	13.83	0.007
ApoB	>90th	265	16.02	14.27	17.78	111	13.07	10.59	15.54	154	18.70	16.00	21.41	<0.001
ApoA1	<10th	160	10.98	9.48	12.47	83	11.41	9.12	13.71	77	10.58	8.36	12.79	0.309
sdLDL-C	>90th	172	10.59	9.11	12.06	71	9.42	7.27	11.58	101	11.64	9.32	13.97	0.080
Non-HDL-C	>90th	270	15.46	13.74	17.19	104	11.69	9.31	14.07	166	18.89	16.15	21.63	<0.001

Pth, percentile; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1; sdLDL-C, small dense low-density lipoprotein; Non-HDL-C, non-high-density lipoprotein cholesterol; CI, confidence interval.

[a]Comparison between men and women; Statistical significance (P <0.05).

3.1.2.2. Analysis of the prevalence of extreme dyslipidaemia (95th or 5th percentiles)

The estimated prevalence of very high (P95th) and very low (P5th) lipid levels in the Portuguese population was also analysed and is presented in Tables 2 and 3. About 10.86 % (7.79% for men and 13.64% for women), 11.18% (9.02% for men and 13.13% for women), and 7.96% (8.07% for men and 7.87% for women) were above the P95th, presenting very high TC, LDL-C and TG levels, respectively. In addition, 11.10% (10.10% for men and 12.00% for women), 9.40% (7.34% for men and 11.27% for women), and 6.36% (5.05% for men and 7.55% for women) were above the P95th, presenting very high apoB, non-HDL-C, and sdLDL-C phenotype, respectively. When analysing very low phenotype for HDL-C, 5.49% (4.14% for men and 6.70% for women) were below the P5th, and for apoA1, 7.30% (8.10% for men and 6.57% for women) were below the P5th.

Table 2 – Estimated prevalence of individuals above the 95th percentile.

	Prevalence of individuals above the 95th percentile											
	Total				Men				Women			
Lipid biomarker	**n**	%	95% CI		**n**	%	95% CI		**n**	%	95% CI	
Total cholesterol	191	10.86	9.37	12.35	68	7.79	5.85	9.74	123	13.64	11.29	15.99
LDL-C	185	11.18	9.67	12.69	72	9.02	6.88	11.16	113	13.13	10.79	15.48
HDL-C	88	5.52	4.43	6.62	38	5.31	3.62	7.01	50	5.72	4.08	7.35
Triglycerides	119	7.96	6.67	9.26	61	8.07	6.06	10.08	58	7.87	5.93	9.80
ApoB	176	11.10	9.59	12.60	82	10.10	7.87	12.33	94	12.00	9.73	14.27
ApoA1	83	5.63	4.53	6.74	37	5.14	3.49	6.79	46	6.08	4.32	7.83
sdLDL-C	98	6.36	5.19	7.53	37	5.05	3.46	6.65	61	7.55	5.62	9.48
Non-HDL-C	158	9.40	8.00	10.80	60	7.34	5.46	9.23	98	11.27	9.05	13.48

LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1; sdLDL-C, small dense low-density lipoprotein; Non-HDL-C, non-high-density lipoprotein cholesterol; CI, confidence interval.

Table 3 – Estimated prevalence of individuals below the 5th percentile.

	Prevalence of individuals below the 5th percentile											
	Total				Men				Women			
Lipid biomarker	n	%	95% CI		n	%	95% CI		n	%	95% CI	
Total cholesterol	68	4.38	3.40	5.35	44	5.01	3.55	6.47	24	3.80	2.42	5.18
LDL-C	66	3.96	3.03	4.89	42	4.38	2.99	5.78	24	3.57	2.26	4.88
HDL-C	79	5.49	4.39	6.58	30	4.14	2.72	5.57	49	6.70	4.91	8.50
Triglycerides	65	4.20	3.25	5.16	38	4.82	3.21	6.43	27	3.64	2.29	4.98
ApoB	69	3.58	2.69	4.47	35	3.63	2.34	4.92	34	3.54	2.23	4.85
ApoA1	107	7.30	6.06	8.54	61	8.10	6.18	10.03	46	6.57	4.83	8.31
sdLDL-C	87	5.08	4.04	6.13	47	5.89	4.15	7.62	40	4.35	3.01	5.70
Non-HDL-C	61	3.89	2.97	4.82	38	4.19	2.80	5.57	23	3.63	2.25	5.00

LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1; sdLDL-C, small dense low-density lipoprotein; Non-HDL-C, non-high-density lipoprotein cholesterol; CI, confidence interval.

3.1.2.3. Prevalence of individuals with values above the 50th percentile for TC and LDL-C

Although in this study dyslipidaemia has been considered only for values >P90th (at risk values) for better comparison between studies the prevalence of individuals with values above P50th for TC and LDL-C was also calculated. The prevalence of individuals having TC values above the P50th was as follows: 55.47% CI=[53.11%-57.84%] (n=963), 59.03% CI=[55.56%-62.49%] (n=503) for women, and 51.56% CI=[47.94%-55.18%](n=460) for men. The prevalence of individuals having LDL-C values above the P50th was as follows: LDL-C: 54.22% CI=[51.85%-56.84%] (n=924), 59.47% CI=[56.02%-62.92%] (n=503) for women and 48.43% CI=[44.82%-52.03%] (n=421) for men. Results for the remaining lipid biomarkers are presented in Table 4.

Table 4 – Estimated prevalence of individuals above or below the 50th percentile.

		Prevalence of individuals above or below the 50th percentile												
		Total				Men				Women				
Lipid biomarker	**Percentile**	**n**	%	95% CI		**n**	%	95% CI		**n**	%	95% CI		P value[a]
Total cholesterol	>50th	963	55.47	53.11	57.84	460	51.56	47.94	55.18	503	59.03	55.56	62.49	<0.001
LDL-C	>50th	924	54.22	51.85	56.58	421	48.43	44.82	52.03	503	59.47	56.02	62.92	<0.001
HDL-C	<50th	830	50.95	48.56	53.34	434	51.95	48.33	55.58	396	50.04	46.51	53.57	0.242
Triglycerides	>50th	884	51.89	49.50	54.28	440	53.36	49.73	56.99	444	50.56	47.40	53.71	0.136
ApoB	>50th	982	56.87	54.51	59.23	465	52.91	49.31	56.51	517	60.46	57.05	63.87	<0.001
ApoA1	<50th	787	48.97	46.58	51.35	427	51.98	48.33	55.62	360	46.24	42.75	49.73	0.023
sdLDL-C	>50th	825	48.28	45.90	50.67	398	46.46	42.84	50.09	427	49.94	46.39	53.48	0.134
Non-HDL-C	>50th	965	55.51	53.15	57.88	455	51.30	47.68	54.91	510	59.34	55.90	62.77	<0.001

Pth, percentile; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1; sdLDL-C, small dense low-density lipoprotein; Non-HDL-C, non-high-density lipoprotein cholesterol; CI, confidence interval.

[a]Comparison between men and women; Statistical significance (P <0.05).

3.1.2.4. Analysis of dyslipidaemia versus treatment

From all individuals under lipid-lowering therapy for hypercholesterolaemia (22.90% CI=[20.98%-25.01%] (n=454)), only 20.69% (n=30) of the individuals in our sample that were calculated to be above the P90th prior to medication reached TC values below the P50th; for LDL-C was it even less (14.49% (n=20)) and 10.56% (n=15) for apoB. The number of men and women under medication is very similar (22.33% CI=[19.73%-24.93%] (n=228) and 23.60% CI=[21.06%-26.14%] (n=226), respectively). However, considering all medicated individuals (n=454) the number of women with values above the P90th is higher: for TC: 42.04% CI=[35.60%-48.47%] (n=95/226) women *versus* 21.93% CI=[16.56%-27.30%] (n=50/228) men; LDL-C: 36.73% CI=[30.44%-43.01%] (n=83/226) women *versus* 24.12% CI=[18.57%-29.68%] (n=55/228) men; apoB: 40.27% CI=[33.87%-46.66%] (n=91/226) women *versus* 22.37% CI=[19.96%-22.78%] (n=51/228) men. In this group, more women than men tend to reach the desirable values (<P50th): TC: 28.42% CI=[19.35%-37.49%] (n=27/95) women *versus* 6.00% CI=[-0.58%-12.58%] (n=3/50) men; LDL-C: 18.07% CI=[9.79%-26.35%] (n=15/83) women *versus* 9.09% CI=[1.49%-16.69%] (n=5/55) men; apoB: 15.39% CI=[7.97%-22.80%] (n=14/91) women *versus* 1.96% CI=[1.84%-5.77%] (n=1/51) men. Additionally, the estimated prevalence of not medicated individuals with TC, LDL-C, and apoB levels above the P90th was 10.26%, 9.39%, and 10.13%, respectively (Table 5).

Table 5 – Estimated prevalence of not medicated individuals above the 90th percentile.

	Prevalence of individuals not medicated above the 90th percentile											
	Total				Men				Women			
Lipid biomarker	**n**	%	95% CI		**n**	%	95% CI		**n**	%	95% CI	
Total cholesterol	140	10.26	8.56	11.96	65	8.71	6.43	10.98	75	11.67	8.99	14.35
LDL-C	124	9.39	7.76	11.03	57	8.20	5.92	10.48	67	10.47	7.93	13.02
ApoB	130	10.13	8.44	11.82	63	9.18	6.79	11.57	67	10.99	8.38	13.61

LDL-C, low-density lipoprotein cholesterol; ApoB, apolipoprotein B; CI, confidence interval.

3.1.2.5. Dyslipidaemia and other cardiovascular risk factors

When analysing the aggregation of one lipid biomarker above the P95th and the presence of at least one additional cardiovascular risk factor, such as HT, diabetes, overweight/obesity, smoking, high alcohol intake, and family history of pCVD or hypercholesterolaemia, the prevalence determined was as follow: TC (10.13%), LDL-C (10.25%), TG (7.66%), non-HDL-C (8.64%), apoB (10.63%), sdLDL-C (5.72%) (Table 6). Among the risk factors listed above, the most prevalent in the individuals with TC, LDL-C, TG, apoB, non-HDL-C or sdLDL-C above the P95th was the overweight/obesity 16.46% CI=[14.69%-18.24%] (n=274) (15.81% CI=[13.12%-18.51%] (n=127) for men and 17.05% CI=[14.49%-19.61%] (n=147) for women), followed by HT, 12.98% CI=[11.37%-14.59%] (n=225) (11.07% CI=[8.79%-13.34%] (n=94) for men and 14.72% CI=[12.39%-17.05%] (n=131) for women).

Table 6 – Estimated prevalence of individuals above the 95th percentile with one additional CVD risk factor.

	Prevalence of individuals above the 95th percentile with one additional CVD risk factor											
	Total				Men				Women			
Lipid biomarker	**n**	%	95% CI		**n**	%	95% CI		**n**	%	95% CI	
Total cholesterol	180	10.13	8.68	11.58	67	7.64	5.71	9.58	113	12.38	10.14	14.63
LDL-C	173	10.25	8.80	11.71	70	8.60	6.50	10.71	103	11.75	9.52	13.98
Triglycerides	114	7.66	6.38	8.93	59	7.64	5.67	9.60	55	7.67	5.76	9.59
ApoB	168	10.63	9.16	12.11	80	9.68	7.49	11.87	88	11.49	9.25	13.73
sdLDL-C	90	5.72	4.60	6.83	34	4.59	3.05	6.12	56	6.74	4.91	8.57
Non-HDL-C	147	8.64	7.29	9.99	58	6.93	5.09	8.77	89	10.20	8.07	12.33

CVD, cardiovascular disease; LDL-C, low-density lipoprotein cholesterol; ApoB, apolipoprotein B; sdLDL-C, small dense low-density lipoprotein; Non-HDL-C, non-high-density lipoprotein cholesterol; CI, confidence interval.

3.1.2.6. Possible monogenic cause of dyslipidaemias

Also, about 3.67% (n=62) of the individuals in our sample had LDL-C or TC above the P95th and family history of pCVD or hypercholesterolaemia, which justify genetic testing for FH. Regarding TG, about 1.84% (n=31) was above the P99th, suggesting the investigation of monogenic disorders associated with extreme levels of TG. On the other

hand, 0.41% (n=7) was below the P1th for apoB and LDL-C, which justify the investigation of hypobetalipoproteinaemia/abetalipoproteinaemia. Additionally, about 0.36% (n=6) presented reduction levels of HDL-C (P1th), and 0.89% (n=15) presented extremely high levels (P99th), and were investigated for variants causing rare genetics forms of HDL-C deficiency.

A total of two pathogenic variants in the *LDLR* gene causing FH were found in 3 individuals from e_COR Study. One variant in the exon 4 (rs387906303), p.(Asp224Asn), and one variant in exon 9 (rs28942079), p.(Ala431Thr). All individuals are heterozygous for these variants, and came from the same region, Algarve.

Of overall 29 individuals that underwent targeted sequencing, all remained after quality control measures. About approximately 430 variants were found for each sample. Across them, we have identified ten missense and one donor splice site DNA sequence variants associated with the phenotypes under study (Supplementary Table 5). All variants were found in heterozygosity. One missense variant in *PCSK9* gene (rs11591147) was found in 1 individual of the P1th of the HDL-C that also presented low levels of LDL-C (P10th-P25th). Additionally, another variant in the *PCSK9* (rs148195424) was identified in 2 individuals from the P1th for LDL-C and apoB. These individuals also presented HDL-C below the P50th. We also found one missense variant in the *ABCG8* gene (rs11887534) in 2 individuals with distinct extreme phenotypes, one in the P99th for TG, and another one in the P1th for LDL-C and apoB. In the *APOB* gene, one rare variant was found (rs72653077) in an individual of the high extreme percentile for HDL-C that also show low LDL-C and apoB levels (P10th-P25th). Still regarding *APOB* gene, one common variant (rs676210) was identified in 10 individuals, 80% (n=8) of these individuals have low levels (P10th-P25th) of LDL-C and/or apoB but also presented other different extreme phenotypes. One variant in the *LCAT* gene (rs4986970) was found in one of these individuals with very low levels of LDL-C and/or apoB, also presenting low HDL-C (P10th-P25th). One missense variant in the *ANGPTL3* gene (rs767910330) was found in 1 individual within P1th for LDL-C and apoB, and another one missense variant was found for the *APOC2* gene (rs120074114) in an individual from the extreme percentile for TG (P99th). For the other 3 individuals in the P99th for TG, two different variants were identified in the *LPL* gene (one for rs1801177, and two for rs268). Still, the variant rs1801177 was also identified in 2 individuals with extreme low and high phenotypes for HDL-C (P1th and P99th, respectively), that presented TG values between P50th and P75th. Lastly, 1 individual in the P99th for TG with also high levels of LDL-C (P90th-P95th) and low levels of HDL-C (P10th-P25th). was found with a silent variant in the

last nucleotide of the exon 8 of the *LIPA* gene (rs116928232) presenting as a functional splice variant.

3.2. Present lipid profile of the Portuguese population

3.2.1. Lipids and lipoproteins

All 1,688 individuals (Supplementary Table 1) were considered for this analysis (except for the apoB (N=1,687) and sdLDL-C (N=1,667)). The present lipid profile of the Portuguese population, by age and gender (Table 7), is as follow in mean values [mg/dL (SD)]: TC=193.89 (SD 38.02) CI=[193.57-194.2]; LDL-C=120.73 (SD 34.30) CI=[120.45-121.01]; TG=108.62 (SD 62.75) CI=[108.10-109.13]; HDL-C=55.77 (SD 15.26) CI=[55.64-55.89]; apoA1=153.39 (SD 30.00) CI=[153.14-153.65]; apoB=93.70 (SD 24.89) CI=[93.50-93.90]; non-HDL-C=138.12 (SD 37.92) CI=[137.81-138.43]; sdLDL-C=29.42 (SD 14.84) CI=[29.25-29.60]. The mean values of TC, LDL-C, TG, apoB, non-HDL-C and sdLDL-C tended to increase and then decreased with age (Table 7). Men in age groups 30-39 and 40-49 had significantly higher LDL-C concentrations than women (P<0.001 and P=0.024, respectively), but older women (50-79) had higher LDL-C than men, although with no statistically significant differences (50-59 (P=0.093), 60-69 (P=0.216), and 70-79 (P=0.869)). When we looked for sdLDL-C particles, the highest concentrations were seen in men for almost all age groups (18-29 (P=0.488), 30-39 (P<0.001), 40-49 (P=0.016), 50-59 (P=0.043), 60-69 (P=0.003) and 70-79 (P<0.001)), and overall (P<<0.001). Looking for apoB, men in age groups 30-39 (P<0.001), 40-49 (P<0.001), 50-59 (P=0.017), and overall (P<0.001) had significantly higher apoB levels than women in the same age range, while for non-HDL-C statistically significant differences were only seen for age groups 30-39 (P<0.001) and 40-49 (P<0.001), and in all ages combined (P=0.002). Concerning TC, concentration levels are not consistent between men and women in age groups 18-29 (P<0.001), 30-39 (P=0.035), 40-49 (P=0.007), 60-69 (0.003), and in all ages combined (P=0.042). For TG, values were higher in men for almost all age groups (18-29 (P=0.123), 30-39 (P=0.010), 40-49 (P<0.001), 50-59 (P=0.010), 60-69 (P=0.010), and 70-79 (P=0.148)) and overall (P<0.001). For HDL-C and apoA1, the profile of serum concentrations is similar, being lower in men with statistically significant differences for all age groups (18-29 (P<<0.001), 30-39 (P<<0.001), 40-49 (P<0.001), 50-59 (P=0.005), 60-69 (P<0.001), and 70-79 (P<0.001) in HDL-C; (18-29 (P<<0.001), 30-39 (P<<0.001), 40-49 (P<0.001), 50-59 (P=0.017), 60-69 (P<0.001), and 70-79 (P<0.001) in apoA1) and for all ages combined (P<<0.001 for HDL-C and apoA1).

3.2.2. Atherogenic risk

It was the first time that apoB and sdLDL-C biomarkers were analysed with the objective to be representative for the Portuguese population, so these lipid biomarkers were analysed with more detail in the present study. Characteristics of individuals with desirable and high concentrations of apoB and sdLDL-C, according to reference values estimated for the Portuguese population were presented in Table 8.

Men and women with high concentrations of apoB or sdLDL-C had higher levels of glucose, TC, LDL-C, HDL-C, TG, and non-HDL-C, than individuals with low apoB or sdLDL-C, showing a very similar profile. Also, BMI, DBP and SBP increase when apoB or sdLDL-C is increasing. In contrast, HDL-C and apoA1 tended to decrease, despite no great differences. Looking for the association of apoB and sdLDL-C, both increase in the same direction with great evidence. Concerning alcohol intake, concentration of apoB and sdLDL-C also increases when alcohol concentration is increasing, and the consumption of alcohol by men is much higher than by women. The same profile was observed for TG levels, which are greater in men than in women even in the highest percentiles (above the $P90^{th}$).

The relation between apoB and sdLDL-C with other lipid and non-lipid risk factors was also analysed by Pearson correlation and are presented in Table 9, showing a very similar profile between these two lipid biomarkers. ApoB and sdLDL-C was significantly correlated ($P<0.001$) with age, DBP, SBP, BMI, alcohol intake and glucose. As expected, the correlation between apoB and sdLDL-C with other lipid risk factors, namely TC, LDL-C, TG, non-HDL-C, and also apoB and sdLDL-C with each other, was significantly and very marked. Also, apoB and sdLDL-C was inversely correlated with HDL-C ($P<<0.001$).

Table 7 – Lipid and lipoprotein mean values by gender and age group.

			Total cholesterol		LDL-C	
			Mean (SD)	95% CI	Mean (SD)	95% CI
Gender	**Age group**	**N**	mg/dL			
	18-29	125	167.61 (32.40)	163.98-171.24	103.92 (29.08)	100.67-107.18
	30-39	171	197.01 (37.90)	193.65-200.37	129.34 (32.61)	126.43-132.26
	40-49	96	202.63 (34.87)	198.70-206.56	132.49 (31.26)	129.13-135.85
Men	50-59	100	206.20 (38.06)	202.45-209.95	134.64 (36.09)	131.08-138.21
	60-69	191	189.96 (39.26)	186.79-193.13	118.21 (36.81)	115.22-121.20
	70-79	165	188.51 (47.15)	184.02-193.00	117.25 (40.85)	113.37-121.13
	18-79	848	192.60 (39.94)	191.97-193.23	123.46 (35.77)	122.89-124.02
	18-29	154	180.74 (31.73)	177.55-183.93	100.62 (26.25)	97.97-103.26
	30-39	162	186.14 (30.85)	183.14-189.14	110.22 (25.08)	107.80-112.64
	40-49	107	197.90 (38.52)	193.41-202.38	120.85 (34.62)	116.84-124.85
Women	50-59	100	206.41 (37.25)	201.91-210.91	132.67 (35.31)	128.45-136.88
	60-69	176	204.32 (35.75)	201.24-207.41	126.16 (32.24)	123.38-128.93
	70-79	141	194.12 (34.21)	190.99-197.25	117.97 (30.35)	115.23-120.71
	18-79	840	195.06 (36.14)	194.44-195.68	118.25 (32.72)	117.71-118.0
	18-29	279	174.15 (32.73)	171.50-176.80	102.28 (27.76)	99.96-104.50
	30-39	333	191.40 (34.87)	189.08-193.72	119.47 (30.50)	117.49-121.46
	40-49	203	200.18 (36.89)	196.97-203.38	126.46 (33.55)	123.66-129.25
Total	50-59	200	206.31 (37.64)	202.96-209.67	133.61 (35.70)	130.48-136.73
	60-69	367	197.69 (38.09)	195.40-199.98	122.48 (34.66)	120.35-124.62
	70-79	306	191.71 (40.39)	189.19-194.22	117.66 (35.26)	115.48-119.84
	18-79	1,688	193.89 (38.02)	193.57-194.20	120.73 (34.30)	120.45-121.01

			HDL-C		Triglycerides	
			Mean (SD)	95% CI	Mean (SD)	95% CI
Gender	**Age group**	**N**	mg/dL			
	18-29	125	50.22 (11.49)	49.02-51.42	86.43 (47.87)	81.44-91.42
	30-39	171	47.83 (14.79)	46.56-49.11	120.43 (82.88)	113.76-127.09
	40-49	96	49.69 (14.40)	48.03-51.34	126.50 (70.59)	117.91-135-08
Men	50-59	100	50.92 (14.69)	49.18-52.66	133.27 (78.64)	124.49-142.05
	60-69	191	51.39 (13.54)	50.28-52.51	126.77 (65.94)	121.41-132.13
	70-79	165	52.14 (13.36)	50.90-53.38	125.10 (71.47)	118.29-131.91
	18-79	848	50.16 (13.89)	49.92-50.41	119.33 (72.44)	118.12-120.55
	18-29	154	65.75 (13.63)	64.42-67.07	85.85 (39.07)	81.81-89.90
	30-39	162	61.43 (13.83)	60.09-67.07	86.54 (37.16)	82.95-90.13
	40-49	107	61.6 (15.070)	59.84-63.37	97.71 (58.13)	90.77-104.65
Women	50-59	100	56.73 (14.63)	55.02-58.44	102.72 (49.44)	96.74-108.71
	60-69	176	59.22 (13.25)	58.05-60.39	110.03 (48.45)	105.61-114.46
	70-79	141	60.58 (16.06)	59.12-62.04	115.87 (61.67)	110.12-121.62
	18-79	840	60.85 (14.65)	60.60-61.10	98.90 (50.51)	98.02-99.77
	18-29	279	57.95 (14.80)	56.94-58.96	86.14 (43.71)	82.35-89.93
	30-39	333	54.85 (15.83)	53.85-55.85	102.94 (65.75)	98.33-107.54
	40-49	203	55.86 (15.91)	54.56-57.16	111.58 (66.02)	105.60-117.55
Total	50-59	200	53.96 (14.95)	52.70-55.22	117.29 (66.79)	111.69-122.90
	60-69	367	55.60 (13.94)	54.78-56.42	117.77 (57.80)	114.26-121.28
	70-79	306	56.95 (15.53)	56.04-57.86	119.84 (66.22)	115.69-124.00
	18-79	1,688	55.77 (15.26)	55.64-55.89	108.62 (62.75)	108.10-109.13

Continuation of Table 7 – Lipid and lipoprotein mean values by gender and age group.

Gender	Age group	N	ApoB (n=1,686) Mean (SD)	ApoB (n=1,686) 95% CI	ApoA1 Mean (SD)	ApoA1 95% CI
			mg/dL			
Men	18-29	125	78.03 (21.18)	74.70-80.35	135.01 (19.67)	132.92-137.10
	30-39	171	98.62 (24.71)	96.47-100.77	137.82 (26.09)	135.61-140.03
	40-49	96	103.58 (24.43)	100.88-106.28	144.75 (27.99)	141.30-148.21
	50-59	100	106.52 (25.72)	103.72-109.32	146.37 (25.99)	143.28-149.46
	60-69	191	97.13 (25.34)	95.09-99.17	147.49 (25.09)	145.40-149.58
	70-79	165	95.17 (28.91)	92.46-97.89	148.47 (26.72)	145.95-150.99
	18-79	848	96.77 (26.58)	96.35-97.20	142.79 (25.86)	142.34-143.25
Women	18-29	154	78.52 (18.50)	76.68-80.35	170.01 (30.49)	167.04-172.97
	30-39	162	84.70 (18.49)	82.95-86.45	165.01 (31.38)	161.83-168.19
	40-49	107	92.69 (23.44)	90.12-95.26	165.65 (37.95)	161.35-169.95
	50-59	100	100.05 (26.09)	98.86-103.24	155.38 (28.25)	152.05-158.72
	60-69	176	96.68 (21.14)	94.84-98.52	160.31 (19.48)	158.21-162.42
	70-79	141	92.71 (20.85)	90.80-94.62	161.47 (25.71)	159.13-163.80
	18-79	840	90.91 (22.89)	90.53-91.29	163.01 (30.25)	162.47-163.55
Total	18-29	279	78.27 (19.89)	76.57-79.91	152.43 (31.03)	150.54-154.33
	30-39	333	91.43 (22.81)	89.93-92.94	151.85 (31.98)	149.91-153.79
	40-49	203	97.94 (24.53)	95.87-100.00	155.58 (35.12)	152.85-158.32
	50-59	200	103.14 (26.12)	100.85-105.42	151.08 (27.56)	148.76-153.42
	60-69	367	96.89 (23.18)	95.44-98.33	154.39 (23.15)	152.90-155.88
	70-79	306	93.77 (24.67)	92.22-95.01	155.87 (26.93)	154.26-157.49
	18-79	1,688	93.70 (24.89)	93.50-93.90	153.39 (30.00)	153.14-153.65

Gender	Age group	N	sdLDL-C (n=1,667) Mean (SD)	sdLDL-C (n=1,667) 95% CI	Non-HDL-C Mean (SD)	Non-HDL-C 95% CI
			mg/dL			
Men	18-29	125	21.81 (9.74)	17.35-26.27	117.39 (32.54)	113.76-121.03
	30-39	171	32.63 (15.67)	30.91-34.35	149.18 (38.50)	145.74-152.62
	40-49	96	35.01 (16.90)	33.06-36.96	152.94 (35.80)	148.96-156.92
	50-59	100	39.61 (18.50)	36.96-42.25	155.28 (38.24)	151.34-159.22
	60-69	191	33.34 (15.92)	32.04-34.63	138.57 (37.05)	135.42-141.72
	70-79	165	32.34 (15.42)	30.89-33.79	136.37 (43.47)	132.26-140.47
	18-79	848	32.51 (16.59)	32.10-32.92	142.43 (39.60)	141.80-143.10
Women	18-29	154	22.05 (9.93)	21.10-23.00	114.99 (29.34)	112.01-117.98
	30-39	162	23.81 (10.24)	20.15-27.47	124.71 (27.51)	122.07-127.35
	40-49	107	27.65 (13.90)	26.10-29.19	136.29 (39.19)	131.84-140.75
	50-59	100	30.43 (15.40)	28.48-32.38	149.68 (37.06)	145.22-154.14
	60-69	176	28.11 (9.95)	27.23-29.00	145.11 (34.93)	142.07-148.14
	70-79	141	27.70 (11.04)	26.69-28.71	133.54 (33.02)	130.55-136.53
	18-79	840	26.63 (12.40)	26.32-26.93	134.20 (35.88)	133.61-134.80
Total	18-29	279	21.10 (10.53)	20.28-21.93	116.20 (31.01)	113.59-118.81
	30-39	333	27.10 (14.60)	24.19-30.01	136.55 (35.46)	134.27-138.84
	40-49	203	31.19 (15.85)	29.78-32.60	144.31 (38.51)	141.03-147.59
	50-59	200	34.81 (17.56)	33.19-36.42	152.35 (37.73)	149.03-155.68
	60-69	367	30.53 (13.31)	29.69-31.37	142.09 (36.07)	139.80-144.37
	70-79	306	29.70 (13.30)	28.87-30.53	134.76 (37.90)	132.39-137.13
	18-79	1,688	29.10 (15.14)	29.25-29.60	138.12 (37.92)	137.81-138.43

LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1; sdLDL-C, small dense low-density lipoprotein; Non-HDL-C, non-high-density lipoprotein cholesterol; SD, standard deviation; CI, confidence interval.

Table 8 – Characteristics of individuals with normal, high and very high apolipoprotein B, and small dense LDL-C levels for men, women, and total.

	sdLDL-C (N=839)					ApoB (N=847)				
	<50th	**≥50th<90th**	**≥50th<95th**	**≥90th<95th**	**≥95th**	**<50th**	**≥50th<90th**	**≥50th<95th**	**≥90th<95th**	**≥95th**
Men	< 27 mg/dL	27-51 mg/dL	27-61 mg/dL	51-61 mg/dL	≥ 61 mg/dL	< 93 mg/dL	93-127 mg/dL	93-142 mg/dL	127-142 mg/dL	≥ 142 mg/dL
n (%)	346 (41.24)	380 (45.29)	442 (52.68)	62 (7.39)	51 (6.08)	381 (45.41)	357 (42.10)	422 (49.76)	65 (7.67)	44 (51.89)
Age, years (SD)	47.62 (19.95)	53.04 (17.12)	53.62 (16.77)	57.28 (13.98)	53.86 (14.58)	48.37 (20.41)	52.63 (16.25)	53.00 (16.20)	55.06 (15.90)	54.96 (14.49)
BMI, kg/m^2 (SD)	25.93 (4.28)	27.72 (4.16)	27.91 (4.15)	29.07 (3.92)	29.61 (3.52)	26.05 (4.54)	27.93 (3.99)	28.01 (3.97)	28.48 (3.90)	28.73 (3.37)
Smokers, n (%)	95 (27.46)	106 (27.89)	123 (27.82)	17 (27.42)	11 (21.57)	102 (26.77)	104 (29.13)	119 (28.20)	15 (23.08)	10 (22.72)
Alcohol intake, g/L (SD)	11.70 (14.21)	17.94 (19.97)	18.87 (20.87)	24.66 (25.49)	22.69 (19.67)	12.70 (14.80)	18.29 (19.97)	18.16 (20.51)	17.46 (23.44)	26.54 (24.42)
SBP, mmHg (SD)	128.74 (18.59)	136.95 (21.00)	137.54 (20.76)	141.30 (18.84)	143.28 (22.27)	130.33 (20.34)	135.84 (19.49)	136.22 (19.72)	138.26 (21.01)	147.42 (21.22)
DBP, mmHg (SD)	78.31 (10.74)	83.43 (10.75)	83.90 (10.69)	86.80 (9.91)	85.49 (11.45)	78.72 (11.32)	83.33 (9.79)	83.48 (9.96)	84.26 (10.89)	88.92 (12.98)
Glucose, mg/dL (SD)	98.16 (26.79)	100.55 (23.65)	100.99 (24.16)	103.74 (27.20)	116.84 (48.31)	99.04 (27.04)	101.18 (25.29)	101.53 (26.46)	103.48 (32.31)	107.00 (38.84)
Total cholesterol, mg/dL (SD)	165.04 (29.25)	205.97 (27.49)	208.89 (29.07)	227.33 (32.16)	259.02 (34.63)	162.21 (24.98)	206.68 (21.91)	212.46 (26.10)	244.20 (16.27)	275.68 (28.08)
LDL-C, mg/dL (SD)	97.84 (25.57)	134.23 (24.80)	136.74 (26.18)	152.60 (29.15)	180.04 (35.10)	93.91 (20.16)	135.37 (17.43)	140.68 (20.92)	169.82 (12.99)	199.98 (23.95)
HDL-C, mg/dL (SD)	52.04 (13.07)	50.50 (13.54)	49.80 (13.30)	45.34 (10.57)	47.55 (14.90)	52.84 (13.76)	49.42 (13.35)	49.16 (12.87)	47.77 (9.80)	46.14 (12.47)
Triglycerides, mg/dL (SD)	87.45 (42.59)	125.86 (63.74)	134.21 (69.63)	187.05 (81.79)	213.06 (97.82)	92.18 (49.77)	131.14 (70.91)	135.15 (72.96)	157.19 (80.38)	191.84 (87.61)
ApoB, mg/dL (SD)	76.69 (17.77)	105.14 (16.63)	107.54 (17.92)	122.69 (18.51)	142.30 (20.66)	95.01 (29.09)	105.24 (26.42)	106.70 (26.49)	106.30 (30.22)	107.41 (32.46)
ApoA1, mg/dL (SD)	141.29 (22.94)	146.69 (25.21)	146.33 (24.79)	144.04 (21.97)	148.63 (29.14)	144.00 (24.39)	145.54 (25.27)	145.28 (24.50)	143.88 (19.85)	140.52 (25.68)
sdLDL-C, mg/dL (SD)	19.00 (4.61)	35.37 (6.12)	38.13 (8.80)	54.69 (2.55)	72.27 (11.25)	28.34 (14.70)	31.72 (14.25)	31.96 (14.15)	32.64 (15.48)	33.07 (15.98)
Non-HDL-C, mg/dL (SD)	113.00 (26.96)	155.47 (26.13)	159.09 (28.05)	181.98 (29.17)	211.47 (33.49)	109.37 (20.91)	157.27 (18.87)	163.30 (22.97)	196.43 (13.16)	229.55 (21.91)
ApoB/apoA1, mg/dL (SD)	0.55 (0.16)	0.74 (0.19)	0.75 (0.20)	0.87 (0.20)	0.99 (0.26)	0.61 (0.22)	0.67 (0.21)	0.67 (0.21)	0.69 (0.22)	0.70 (0.22)
sdLDL-C/LDL-C, mg/dL (SD)	0.20 (0.06)	0.28 (0.06)	0.29 (0.08)	0.38 (0.11)	0.41 (0.08)	0.23 (0.09)	0.28 (0.08)	0.28 (0.08)	0.30 (0.08)	0.33 (0.09)

Continuation of Table 8 – Characteristics of individuals with normal, high and very high apolipoprotein B, and small dense LDL-C levels for men. women, and total.

	sdLDL-C (N=828)					ApoB (N=839)				
	<50th	**≥50th<90th**	**≥50th<95th**	**≥90th<95th**	**≥95th**	**<50th**	**≥50th<90th**	**≥50th<95th**	**≥90th<95th**	**≥95th**
Women	< 23 mg/dL	23-39 mg/dL	23-42 mg/dL	39-42 mg/dL	≥ 42 mg/dL	< 79 mg/dL	79-114 mg/dL	79-130 mg/dL	114-130 mg/dL	≥ 130 mg/dL
n (%)	346 (41.79)	366 (44.20)	402 (48.55)	36 (4.35)	80 (9.67)	271 (32.20)	455 (54.23)	528 (62.93)	73 (8.70)	40 (4.77)
Age, years (SD)	45.72 (18.92)	52.37 (17.81)	52.27 (17.56)	51.33 (15.27)	51.42 (16.60)	43.04 (18.76)	51.48 (17.68)	52.12 (17.55)	56.10 (16.26)	54.38 (14.40)
BMI, kg/m^2 (SD)	25.09 (4.89)	27.53 (5.51)	27.47 (5.44)	26.94 (4.81)	28.90 (5.20)	24.87 (4.86)	27.18 (5.41)	27.40 (5.40)	28.78 (5.16)	27.76 (5.14)
Smokers, n (%)	65 (18.79)	57 (15.57)	62 (15.42)	5 (13.88)	14 (17.50)	52 (19.19)	76 (16.70)	85 (16.10)	9 (12.33)	7 (17.50)
Alcohol intake, g/L (SD)	2.76 (6.16)	3.18 (7.70)	3.07 (7.48)	2.11 (5.04)	4.22 (7.30)	2.94 (5.90)	2.98 (7.63)	3.04 (7.40)	3.46 (5.90)	3.33 (5.92)
SBP, mmHg (SD)	116.69 (21.93)	125.55 (22.24)	125.82 (22.19)	128.24 (21.77)	131.13 (22.68)	114.14 (20.35)	124.35 (22.24)	125.77 (22.61)	134.60 (23.11)	132.58 (24.18)
DBP, mmHg (SD)	74.76 (10.73)	80.09 (10.89)	80.22 (10.86)	81.40 (10.63)	83.81 (12.78)	74.00 (10.58)	79.07 (10.50)	79.79 (10.93)	84.25 (12.48)	85.36 (14.32)
Glucose, mg/dL (SD)	88.21 (14.71)	94.19 (22.10)	94.04 (21.79)	92.75 (19.04)	101.92 (34.55)	87.75 (16.80)	92.79 (20.11)	93.61 (20.51)	98.71 (22.31)	101.20 (38.36)
Total cholesterol, mg/dL (SD)	172.69 (25.79)	204.87 (27.86)	207.79 (29.43)	233.58 (30.64)	237.90 (35.98)	164.27 (20.75)	199.87 (22.04)	204.73 (24.92)	235.00 (20.10)	276.22 (34.04)
LDL-C, mg/dL (SD)	96.34 (21.83)	124.96 (25.33)	127.77 (26.94)	152.65 (28.21)	155.72 (34.03)	86.06 (14.06)	121.45 (17.97)	126.34 (21.41)	156.86 (14.96)	192.45 (28.61)
HDL-C, mg/dL (SD)	62.67 (15.13)	61.76 (14.19)	61.44 (14.10)	58.68 (13.14)	56.76 (13.72)	64.89 (15.89)	60.63 (13.80)	60.12 (13.74)	56.96 (12.99)	58.03 (13.12)
Triglycerides, mg/dL (SD)	75.99 (28.92)	105.36 (42.98)	107.02 (43.82)	121.725 (48.80)	158.36 (67.21)	76.32 (30.92)	102.11 (45.61)	105.57 (46.49)	127.11 (46.38)	143.40 (76.61)
ApoB, mg/dL (SD)	74.64 (15.01)	95.51 (15.01)	97.68 (16.30)	116.73 (14.90)	121.07 (22.21)	92.14 (27.88)	97.10 (22.86)	99.46 (22.95)	100.62 (26.59)	105.45 (32.87)
ApoA1, mg/dL (SD)	158.96 (27.52)	168.22 (28.66)	168.24 (28.20)	168.43 (24.09)	168.18 (34.36)	164.59 (30.14)	164.44 (28.69)	164.13 (28.44)	162.14 (26.89)	162.18 (26.00)
sdLDL-C, mg/dL (SD)	16.91 (3.99)	28.91 (4.42)	29.99 (5.46)	40.22 (0.80)	50.65 (10.80)	25.10 (11.34)	27.28 (11.06)	28.42 (11.72)	28.21 (12.41)	30.25 (15.37)
Non-HDL-C, mg/dL (SD)	110.02 (23.61)	143.11 (25.82)	146.35 (27.59)	174.90 (26.49)	181.14 (34.88)	99.39 (15.06)	139.24 (18.46)	144.61 (22.38)	178.04 (14.23)	218.20 (30.54)
ApoB/apoA1, mg/dL (SD)	0.49 (0.13)	0.58 (0.15)	0.59 (0.17)	0.70 (0.13)	0.75 (0.22)	0.55 (0.17)	0.59 (0.17)	0.61 (0.18)	0.59 (0.19)	0.62 (0.23)
sdLDL-C/LDL-C, mg/dL (SD)	0.18 (0.04)	0.24 (0.05)	0.25 (0.05)	0.28 (0.06)	0.34 (0.07)	0.21 (0.06)	0.23 (0.07)	0.23 (0.07)	0.25 (0.06)	0.26 (0.09)

Continuation of Table 8 – Characteristics of individuals with normal, high and very high apolipoprotein B, and small dense LDL-C levels for men, women, and total.

	sdLDL-C (N=1,667)					ApoB (N=1,686)				
	<50th	**≥50th<90th**	**≥50th<95th**	**≥90th<95th**	**≥95th**	**<50th**	**≥50th<90th**	**≥50th<95th**	**≥90th<95th**	**≥95th**
Total	< 25 mg/dL	25-44 mg/dL	25-53 mg/dL	44-53 mg/dL	≥ 53 mg/dL	< 88 mg/dL	88-118 mg/dL	88-130 mg/dL	118-130 mg/dL	≥ 130 mg/dL
n (%)	726 (43.55)	711 (42.65)	821 (49.25)	110 (6.60)	119 (7.14)	728 (43.15)	700 (41.49)	828 (49.08)	128 (7.59)	130 (7.71)
Age, years (SD)	46.91 (19.41)	52.37 (17.51)	52.64 (17.36)	54.44 (16.26)	54.52 (13.99)	46.54 (19.80)	52.26 (17.05)	52.60 (16.87)	54.46 (15.80)	54.87 (14.64)
BMI, kg/m^2 (SD)	25.62 (4.83)	27.55 (4.78)	27.70 (4.74)	28.69 (4.39)	29.10 (3.85)	25.67 (4.87)	27.54 (4.77)	27.73 (4.71)	28.75 (4.28)	28.34 (4.32)
Smokers, n (%)	161 (22.18)	156 (21.94)	181 (22.05)	25 (22.73)	28 (23.53)	158 (21.70)	162 (23.14)	189 (22.83)	27 (21.09)	28 (21.54)
Alcohol intake, g/L (SD)	6.68 (11.89)	9.74 (15.39)	10.70 (16.34)	17.00 (20.63)	20.52 (22.71)	7.08 (11.90)	10.25 (15.83)	10.82 (16.59)	13.94 (19.21)	16.18 (22.86)
SBP, mmHg (SD)	122.26 (21.15)	131.16 (22.25)	132.18 (22.26)	138.90 (21.27)	139.71 (20.86)	122.48 (21.39)	130.71 (21.86)	131.59 (21.93)	136.41 (21.80)	139.61 (22.68)
DBP, mmHg (SD)	76.48 (10.70)	81.66 (11.17)	82.16 (11.11)	85.50 (10.09)	86.07 (11.23)	76.59 (10.89)	81.51 (10.78)	81.84 (10.70)	83.63 (10.15)	86.20 (13.02)
Glucose, mg/dL (SD)	92.83 (21.29)	97.93 (25.59)	98.48 (25.55)	102.15 (25.12)	106.43 (35.94)	93.72 (23.84)	97.11 (23.01)	97.55 (22.69)	99.96 (20.76)	104.22 (37.75)
Total cholesterol, mg/dL (SD)	170.13 (28.42)	206.40 (28.63)	208.81 (29.53)	224.81 (30.56)	247.20 (37.81)	165.23 (23.71)	203.91 (21.43)	208.24 (23.47)	231.89 (19.79)	265.74 (29.79)
LDL-C, mg/dL (SD)	97.54 (23.83)	131.05 (25.88)	133.04 (26.56)	146.23 (27.38)	169.21 (35.90)	91.36 (17.43)	129.36 (16.07)	133.79 (19.12)	158.01 (16.14)	187.56 (25.48)
HDL-C, mg/dL (SD)	57.91 (15.29)	56.07 (14.32)	55.51 (14.47)	51.83 (15.00)	47.85 (13.90)	59.26 (15.65)	54.81 (14.46)	54.18 (14.22)	55.73 (12.29)	50.38 (12.83)
Triglycerides, mg/dL (SD)	83.13 (38.34)	112.25 (47.94)	119.09 (56.41)	164.39 (82.07)	199.85 (91.91)	85.35 (40.37)	115.37 (58.27)	119.04 (60.89)	139.10 (70.52)	169.67 (86.26)
ApoB, mg/dL (SD)	75.93 (16.43)	101.08 (16.18)	103.10 (17.14)	116.49 (17.42)	134.15 (22.84)	71.94 (11.69)	101.35 (8.50)	105.93 (11.88)	123.69 (3.33)	150.87 (20.48)
ApoA1, mg/dL (SD)	151.84 (28.29)	157.05 (27.58)	157.02 (28.15)	156.82 (31.84)	149.47 (30.19)	155.61 (29.54)	154.69 (28.30)	154.00 (27.93)	150.22 (25.64)	148.18 (25.60)
sdLDL-C, mg/dL (SD)	18.13 (4.36)	32.38 (5.28)	34.37 (7.11)	47.18 (2.77)	64.61 (11.37)	19.89 (6.94)	29.96 (9.48)	31.40 (10.46)	37.05 (12.12)	47.55 (16.82)
Non-HDL-C, mg/dL (SD)	112.22 (25.70)	150.33 (26.76)	153.30 (27.84)	172.98 (26.94)	199.35 (35.69)	105.97 (18.34)	149.10 (16.92)	154.06 (20.23)	181.16 (14.58)	215.36 (25.63)
ApoB/apoA1, mg/dL (SD)	0.52 (0.15)	0.66 (0.17)	0.68 (0.18)	0.77 (0.21)	0.93 (0.25)	0.47 (0.12)	0.64 (0.15)	0.66 (0.16)	0.75 (0.17)	0.88 (0.22)
sdLDL-C/LDL-C, mg/dL (SD)	0.19 (0.05)	0.26 (0.06)	0.27 (0.06)	0.34 (0.07)	0.40 (0.10)	0.22 (0.08)	0.25 (0.08)	0.26 (0.08)	0.28 (0.09)	0.30 (0.09)

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1; sdLDL-C, small dense low-density lipoprotein; Non-HDL-C, non-high-density lipoprotein cholesterol; SD, standard deviation.

Percentiles estimated for the Portuguese population were used as reference values.

Age, BMI, alcohol intake, SBP, DBP, glucose, total cholesterol, LDL-C, HDL-C, triglycerides, apoB, apoA1, non-HDL-C, sdLDL-C, apoB/apoA1, and sdLDL-C/LDL-C are presented as mean (SD).

Table 9 – Pearson correlations among lipid and non-lipid risk factors.

	Men and Women (N=1,688)															
	Total cholesterol		**Triglycerides**		**LDL-C**		**HDL-C**		**ApoB**		**ApoA1**		**Non-HDL-C**		**sdLDL-C**	
Variable	*r*[a]	P value	*r*[a]	P value	*r*[a]	P value	*r*[a]	P value	*r*[a]	P value	*r*[a]	P value	*r*[a]	P value	*r*[a]	P value
Age, years	0.356	<<0.001[b]	0.160	<<0.001[b]	0.358	<<0.001[b]	-0.022	0.459	0.359	<<0.001[b]	0.028	0.355	0.356	<<0.001[b]	0.268	<<0.001[b]
BMI, kg/m^2	0.226	<<0.001[b]	0.289	<<0.001[b]	0.290	<<0.001[b]	-0.255	<<0.001[b]	0.325	<<0.001[b]	-0.129	0.017[b]	0.325	<<0.001[b]	0.285	<<0.001[b]
Alcohol intake, g/L	0.223	<<0.001[b]	0.137	0.004[b]	0.215	<<0.001[b]	-0.0004	0.990	0.236	<<0.001[b]	0.034	0.254	0.218	<<0.001[b]	0.295	<<0.001[b]
SBP, mmHg	0.317	<<0.001[b]	0.239	<<0.001[b]	0.356	<<0.001[b]	-0.141	<<0.001[b]	0.386	<<0.001[b]	-0.044	0.143	0.366	<<0.001[b]	0.345	<<0.001[b]
DBP, mmHg	0.317	<<0.001[b]	0.263	<<0.001[b]	0.342	<<0.001[b]	-0.129	0.015[b]	0.372	<<0.001[b]	-0.017	0.578	0.361	<<0.001[b]	0.336	<<0.001[b]
Glucose, mg/dL	0.272	<<0.001[b]	0.179	<<0.001[b]	0.303	<<0.001[b]	-0.120	0.060	0.319	<<0.001[b]	-0.044	0.145	0.314	<<0.001[b]	0.301	<<0.001[b]
Total cholesterol, mg/dL	1	<<0.001[b]	0.391	<<0.001[b]	0.920	<<0.001[b]	0.143	0.002[b]	0.875	<<0.001[b]	0.263	<<0.001[b]	0.916	<<0.001[b]	0.708	<<0.001[b]
LDL-C, mg/dL	0.920	<<0.001[b]	0.362	<<0.001[b]	1	<<0.001[b]	-0.182	<<0.001[b]	0.945	<<0.001[b]	-0.062	0.039[b]	0.970	<<0.001[b]	0.721	<<0.001[b]
HDL-C, mg/dL	0.142	0.002[†]	-0.392	<<0.001[b]	-0.182	<<0.001[b]	1	<<0.001[b]	-0.250	<<0.001[b]	0.867	<<0.001[b]	-0.267	<<0.001[b]	-0.171	<<0.001[b]
Triglycerides, mg/dL	0.391	<<0.001[b]	1	<<0.001[b]	0.363	<<0.001[b]	-0.392	<<0.001[b]	0.497	<<0.001[b]	-0.116	<<0.001[b]	0.540	<<0.001[b]	0.567	<<0.001[b]
ApoB, mg/dL (n=1,686)	0.875	<<0.001[b]	0.497	<<0.001[b]	0.945	<<0.001[b]	-0.250	<<0.001[b]	1	<<0.001[b]	-0.077	0.011[b]	0.953	<<0.001[b]	0.768	<<0.001[b]
ApoA1, mg/dL	0.263	<<0.001[b]	-0.116	<<0.001[b]	-0.062	<<0.001[b]	0.867	<<0.001[b]	-0.077	0.011[b]	1	<<0.001[b]	-0.096	<0.001[b]	0.036	0.235
sdLDL-C, mg/dL (n=1,667)	0.708	<<0.001[b]	0.567	<<0.001[b]	0.721	<<0.001[b]	-0.172	<<0.001[b]	0.768	<<0.001[b]	0.036	0.235	0.760	<<0.001[b]	1	<<0.001[b]
Non-HDL-C, mg/dL	0.916	<<0.001[b]	0.540	<<0.001[b]	0.970	<<0.001[b]	-0.267	<<0.001[b]	0.953	<<0.001[b]	-0.096	<0.001[b]	1	<<0.001[b]	0.760	<<0.001[b]

SBP. systolic blood pressure; DBP, diastolic blood pressure; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; sdLDL-C, small dense low-density lipoprotein; Non-HDL-C, non-high-density lipoprotein cholesterol.

[a]Pearson correlation coefficient.

[b]Statistical significance (P value <0.05).

We also examined the atherogenic risk of the Portuguese population by analysing the apoB/apoA1 and sdLDL-C/LDL-C ratios. The mean value for apoB/ApoA1 ratio overall was 0.63 (SD 0.23) CI=[0.634-0.637] (0.70 (SD 0.25) CI=[0.695-0.703] for men and 0.58 (SD 0.19) CI=[0.575-0.581] for women), and if we look for the prevalence accordingly with estimated percentiles for the Portuguese population, about 40.02% CI=[37.70%-42.35%] (n=678) (33.33% CI=[29.92%-36.74%] (n=287) for men and 46.10% CI=[42.63%-49.57%] (n=391) for women) was above the P50th and below the P90th, and 13.39% CI=[11.76%-15.02%] (n=188) (10.60% CI=[8.29%-12.91%] (n=77) for men and 15.92% CI=[13.33%-18.50%] (n=111) for women) was above the P90th for apoB/apoA1 ratio. On the other hand, accordingly to the European Atherosclerosis Society (EAS) reference values, 14.76% CI=[13.07%-16.44%] (n=212) of individuals were under high atherogenic risk, with no statistically significant differences when compared with our reference values (P=0.136). Besides, the prevalence estimated for individuals not following recommendation values for apoB/ApoA1 accordingly to EAS *versus* our reference values (P50th) was 41.09% CI=[38.74%-43.44%] (n=671) and 53.41% CI=[51.07%-55.76%] (n=866), respectively, with statistically significant differences for women (P<0.001) (38.41% CI=[35.10%-41.72%] (n=306) and 62.02% CI=[58.73%-65.31%] (n=502), accordingly to EAS and our reference values, respectively), but not for men (P=0.500) (44.05% CI=[40.51%-47.58%] (n=365) and 43.93% CI=[40.39%-47.46%] (n=364), accordingly to EAS and our reference values, respectively). Regarding the sdLDL-C/LDL-C ratio, mean value for the general population was 0.24 (SD 0.10) CI=[0.238-0.239] (0.26 (SD 0.11) CI=[0.256-0.259] for men and 0.22 (SD 0.07) [0.221-0.223] for women) with prevalence of 45.98% [43.60%-48.37%] (n=838) (44.53% CI=[40.92%-48.15%] (n=410) for men and 47.30% CI=[43.79%-50.82%] (n=428) for women) above the P50th and below the P90th, and 20.35% CI=[18.47%-22.22%] (n=344) (28.52% CI=[25.37%-31.66%] (n=235) for men and 12.93% CI=[10.55%-15.31%] (n=109) for women) above the P90th.

The lipid profile of those individuals above the P90th for sdLDL-C/LDL-C ratio was analysed and showed that mean values for TC, LDL-C, HDL-C, TG, apoB, apoA1, non-HDL-C, and sdLDL-C were above the P75th. In individuals with hypertriglyceridaemia (≥150 mg/dL), the sdLDL-C/LDL-C ratio was statistically significant higher from those with normal TG levels (P<0.001), 0.33 (SD 0.09) CI=[0.32-0.34] *versus* 0.22 (SD 0.07) CI=[0.21-0.23], respectively. Finally, when sdLDL-C/LDL-C ratio was analysed between individuals with and without diabetes, statistically significant differences were seen (P<0.001), presenting higher in the diabetic group 0.28 (SD 0.09) CI=[0.27-0.30] *versus* (0.24 (SD 0.08) CI=[0.23-0.24], respectively).

3.2.3. Atherogenic risk and lipid-lowering therapy

Looking for individuals under lipid-lowering therapy *versus* not medicated, mean values of the medicated group are statistically significantly lower than not medicated for apoB/apoA1, but for sdLDL-C/LDL-C ratio the opposite was observed; 0.60 (SD 0.19) CI=[0.52-0.64] *versus* 0.651 (SD 0.23) CI=[0.649-0.653] for apoB/apoA1 (P=0.009), and 0.27 (SD 0.10) CI=[0.26-0.28] *versus* 0.232 (SD 0.08) CI=[0.231-0.233] for sdLDL-C/LDL-C (P=<0.001), for the medicated *versus* not medicated group, respectively. If we look for the mean values (mg/dL) of apoB and sdLDL-C in those two groups, apoB tended to decrease in the medicated group (91.29 (SD 23.58) CI=[89.16-93.40] *versus* 95.85 (SD 25.45) CI=[95.61-96.08] (P=0.013)), but the same is not observed for sdLDL-C (29.96 (SD 13.34) CI=[28.76-31.16] *versus* 29.47 (SD 14.82) CI=[29.30-29.63] (P=0.145)).

4. DISCUSSION

In this study, we applied for the first time reference values for plasma TC, LDL-C, HDL-C, non-HDL-C, TG, apoA1, apoB, sdLDL-C, as well as for apoB/apoA1 and sdLDL-C/LDL-C ratios based on the lipid percentiles established in our previously study (Bourbon et al., *Submitted*). The highest prevalence of individuals above the P90th for TC, LDL-C, apoB, non-HDL-C and sdLDL-C in the Portuguese population were observed in women, contrasting with previous National studies showing high prevalence of hypercholesterolaemia in men (Instituto de Alimentação Becel, 2002). This evidences the importance of the dermination of reference plasma lipid biochemical biomarkers for gender and age as it was done in this study; general values are used for simplicity at the time of evaluation, but under-diagnoses dyslipidaemia in women.

Although our findings for the prevalence of individuals not following recommendation values for apoB/apoA1 was statistically significant different from EAS, this difference is only because of women's cut-off point, that is lower than previously described (Walldius et al., 2001, 2006).

Like apoB, the non-HDL-C is a measure of the concentration of the total atherogenic lipoproteins in plasma and also a good marker in the cardiovascular risk evaluation (Ramjee et al., 2011; Pencina et al., 2015). Determining the non-HDL-C bring some advantages, such as been less expensive (calculated by simply subtracting HDL-C from TC) and more readily available than measurements of apoA1 and apoB, making this a potential lipid biomarker to be included in the evaluation/characterisation of the dyslipidaemia (Ramjee et al., 2011; Pencina et al., 2015). However, it is often not intuitive for physicians and laboratories to interpret and make decisions upon a non-HDL-C

concentration. By contrast, apoB is not available in all labs, as LDL-C or non-HDL-C, but is actually causative in the progression of atherosclerosis (Tabas, 1997; Epstein and Ross, 1999). Additionally, the measurement of apolipoproteins is not significantly influenced by interference of high TG levels, even by diet, thereby can be dosed with no need of prior fasting (Marcovina and Packard, 2006). Of interest, it has been also proposed the use of apoB/apoA1 ratio as a superior biomarker for predicting cardiovascular risk. For instance, the INTERHEART Study (Yusuf et al., 2004a) mentioned apoB/apoA1 ratio as an important cardiovascular risk marker, and the last guidelines for dyslipidaemia (Catapano et al., 2016) highlight the importance of apoA1 and apoB measurement, but not for diagnosis or as treatment targets. The truth is that the consensus for the prevention of cardiovascular diseases has been the object of controversy and debate among researchers in this area. Today the main objective in all guidelines is LDL-C, being non-HDL-C or ApoB suggested as a secondary targets. According to our results, the lipid-lowering therapy with statins in the apoB is apparently beneficial, and consequently in lowering apoB/apoA1 ratio, suggesting apoB as a good target for therapy with lipid-lowering drugs (Austin et al., 1988; Colhoun et al., 1993; Gotto et al., 2000). It is truth that, if on the one hand, all apoB concentration is the sum of all potential atherogenic particles, on the other hand, in most conditions, more than 90% of all apoB in blood is found in LDL, being total apoB concentration measurement an optimal method for indirect assessing LDL particle number. For instance, in cases where LDL-C is normal or low, high apoB levels may indicate an increased number of sdLDL-C particles. Thus, even lowering-therapy resulting in many patients reaching their LDL-C goal, they can continue to have a high number of sdLDL-C particles and consequently residual risk of vascular events (Sniderman et al., 1980, 1982; Barter et al., 2006; Walldius et al., 2006). In fact, our results showed a very similar profile for both apoB and sdLDL-C, excepted when analysing mean values between medicated and not medicated for cholesterol. The sdLDL-C group did not showed statistically significant difference between them, especially looking for the sdLDL-C/LDL-C ratio, which was higher in the medicated group, reflecting the benefit of the lipid-lowering therapy in the LDL-C or apoB, but not in lowering sdLDL-C. Actually, the effects of statin therapy on LDL subfractions have controversial results and most of our sampled population medicated for cholesterol was under lipid-lowering therapy with statins. Some studies have shown a decrease in the concentration of sdLDL-C (Tilly-Kiesi, 1991; März et al., 2001; Baldassarre et al., 2005; Tokuno et al., 2007; Florentin et al., 2011; Yoshino et al., 2012; Diffenderfer and Schaefer, 2014; Nishikido et al., 2016), whereas others have not (Bredie et al., 1995; Kontopoulos et al., 1996; Rosenson, 2002; Sniderman, 2008). It may be that the type of

stain makes a difference in lowering, or not, the sdLDL; unfortunately this analysis was not possible with our sample. If novel lipid therapies as the PCSK9 inhibitors will indeed reduce sdLDL-C still remains to be seen.

As it has been said apoB and LDL-C are highly correlated since cholesterol is a major component of apoB particles and changes in the apoB particle number of are the major determinants of the levels of cholesterol in plasma (Otvos et al., 2002). This study also support the correlation between apoB and sdLDL-C with other lipid and non-lipid risk factors, such as TC, HDL-C and non-HDL, age, DBP, SBP, BMI, alcohol intake, and glucose, respectively, which evidence the influence of environmental factors and the importance of interaction with other risk factors in the lipid metabolism in modulating the risk.

In another study, Sniderman et al. suggested that apoB measurement maybe useful indicating an increased number of potentially atherogenic lipoprotein, since they identified that 81% of hypertriglyceridaemic patients who survived from a myocardial infarction had elevated apoB, while 70% had normal levels of TG and TC concentrations, but higher levels of apoB (Sniderman et al., 1982). In our study, those presenting high TG or diabetes have high levels for sdLDL-C/LDL-C, showing that even with normal levels of LDL-C, these individuals have atherogenic dyslipidaemia. So, we think it is reasonable to believe that apoB and sdLDL-C measurement will provide valuable information regarding the lipoproteins abnormalities, rather than provided by LDL-C alone.

It is also known that the heterogeneity of the LDL particles with respect to the size and density is an important issue, since small and dense particles have high penetration into the arterial wall, has less affinity to the LDL receptors, and lasts longer in the plasma than the larger particles (Goulinet and Chapman, 1997; Chancharme et al., 1999; Berneis and Krauss, 2002; Lara-Riegos et al., 2013). Therefore, sdLDL-C particles have been suggested as high atherogenic compared with the large LDL particles, making this lipoprotein useful as an atherogenic marker. More than sdLDL-C per se, our study highlights the sdLDL-C/LDL-C ratio as a huge asset to the dyslipidaemia evaluation, by demonstrating that individuals with higher risk levels for this biomarker have general lipid profile above the $P75^{th}$. We suggest that the sdLDL-C/LDL-C ratio should be recommended, particularly in individuals with diabetes or other risk factors (e.g. overweight/obesity, HT, smoking, etc.). Taking all these together, we believe that the present study highlights the importance of the measurement of atherogenic particles, rather than only the cholesterol, and that is essential for an adequate lipid-lowering therapy and/or correct management of environmental factors.

Dyslipidaemia is a disorder that confers high cardiovascular risk. As any disorder based in laboratory values for example diabetes, there are a borderline and at risk values (that define disease) and a reference range can be estimated. Percentiles follow exactly this rationale and values between P50th and P90th are usually considered as borderline values, values above P90th can be considered at risk values. So in this study dyslipidaemia was considered only for values above the P90th (at risk values) and all analysis have been performed based on those values. Nevertheless other studies have considered similar values to the P50th as dyslipidaemia (TC >190 mg/dL and LDL-C >115 mg/dL). Having these values under consideration the present study showed a slight decrease in the prevalence of hypercholesterolaemia (13-17%) comparing with the data presented in the last national study on the lipid profile of the Portuguese population (BECEL Institute, 2001) (Instituto de Alimentação Becel, 2002), in which 68.5% of the Portuguese presented CT values ≥190mg/dL and 71% of LDL-C ≥115mg/dL. However this slight decrease does not follow the exponential increase in the sale of statins in Portugal (4,697,659 packages in 2004 *versus* 9,780,010 in 2013 (CEFAR 2013); 35.0 DHD (Definite Daily Dose/1000 in habitants/day) in 2004 *versus* 96.6 DHD in 2012, representing an increase of 176%, (CEFAR, 2013)).

Finally, with this study we have identified 3 individuals with monogenic cause for FH. Interestingly, the variant p.(Asp224Asn) is one of the most common Portuguese variants and was previously characterised in a British population (Hobbs et al., 1992), and was found in one British individual living in Algarve. This number was expected according to the estimated prevalence for the heterozygous FH (1:500) (Nordestgaard et al., 2013). Also, looking for individuals in the extreme phenotypes for dyslipidaemia lead to the identification of common and rare variants associated to their phenotypes. In fact, the search for variants involved in the modulation of the lipid metabolism has been made possible through the NGS technologies. The two variants of the *PCSK9* (rs11591147 and rs148195424) found in individuals with low cholesterol are well characterised in previous publications, described as associated with increase in the LDLR activity and low levels of LDL-C (Abifadel et al., 2003; Benjannet et al., 2004). Of the two variants identified in *APOB* gene (rs676210 and rsrs72653077), both were already described in patients with hypocholesterolaemia (Leren et al., 1998). Also, the variant found in *LCAT* gene (rs4986970) was associated to HDL-C decrease (Haase et al., 2012), which is in accordance with the phenotype. Interestingly, we have also identified 1 individual in the low percentile for LDL-C/apoB with a variant in the exon 6 of the *ANGPTL3* gene (rs767910330), which corresponds to the C-terminal domain of the protein, known as

involved in angiogenesis (Camenisch et al., 2002) and may be involved in atherosclerosis, since plasma levels are closely associated with arterial wall thickness. Not less interesting, 1 variant in the *ABCG8* gene (rs11887534) was found in 2 individuals with distinct phenotypes, low LDL-C/apoB and high TG, and was previously associated to the susceptibility of gallstone disease (Buch et al., 2007). Finally, of the 4 variants that were found in association with high TG levels, 1 was found in *LIPA* gene (rs116928232) and is a functional mutation causing Lysosomal Acid Lipase Deficiency (LALD) when in homozigosity (Klima et al., 1993), 1 was identified in the *APOC2* gene (rs120074114) and previously described by Hegele et al. (1991) in different types of hyperlipidemic patients (Hegele et al.1991), and the last 2 are common variants of *LPL* gene (rs1801177 and rs268) also found in individuals with mild hypertriglyceridaemia by several studies (Fisher et al., 1997; Sagoo et al., 2008; Martín-Campos et al., 2014; Pirim et al., 2015). Although we have not found any monogenic cause, apart from FH, possible due to the mild phenotype of individuals in the extreme percentiles of our population, the variants identified could explain the dyslipidaemia patterns in these individuals. Despite we did not identify other patients with monogenic dyslipidaemia, the identification of variants in individuals with extremely low or high plasma lipid levels has been successfully (Cohen et al., 2004; Patel et al., 2016; Peloso et al., 2016), and we still believe in this approach of extreme-selected sample as a good strategy to identify genes or variants of interest.

Although dyslipidaemia is a high prevalent risk factor in the Portuguese population, it could be modifiable. The correct and early identification of this CVD risk factor is important for their correct management, and could contribute to CVD prevention, especially changing the life habits. Nevertheless, some of dyslipidaemias have genetic causes (monogenic), being associated with an elevated CVD risk per se, like FH. By contrast, mostly mild to severe dyslipidaemias results from multiple genes with small effect (polygenic dyslipidaemias), as result of various genetic alterations that may interact, increasing or reducing the overall pathogenic effect, while at the same time the final phenotypic expression is being modulated by non-genetic factors. These polygenic dyslipidaemias are more easily modulated by modification of the life habits, and maybe the implementation of lipid-lowering therapy should not be necessary. By contrast, in FH the implementation of lipid-lowering therapy is needed in order to decrease the LDL-C levels and the CVD risk. Understanding the effects of associations and/or interactions between genetic, non-genetic and environmental risk factors in lipid metabolism, should contribute to enlarge the knowledge of the lipid metabolism and its relation to CVD risk.

5. CONCLUSIONS

Although high values of dyslipidaemia have been found, dyslipidaemia is a modifiable cardiovascular risk factor, so changes in life styles and health policies must be made. Dyslipidaemia in woman have been under-diagnosed due to lack of gender specific reference values and this has to be urgently addressed since cardiovascular disease is increasing in woman. Fortunately the woman that have been diagnosed and treated comply with the medication and/or life style recommendations; the same is not true for men whom we have shown to have a worse compliance with the prescribed medication, and must probably with life style changes.

Taking all the data presented in consideration, we believe that the present study highlights the importance of the measurement of atherogenic particles, rather than only the cholesterol, and that these measurements are essential for an adequate lipid-lowering therapy and/or correct management of environmental factors. The work also adds evidence to an on going debate about if statins lower or not sdLDL; in this study statin use was associated to a decrease in apoB levels but not in sdLDL-C, also shown by individuals medicated having a higher sdLDL-C/LDL-C ratios than the ones not under medication.

Although statin prescription has increased greatly in the last 10 years the dyslipidaemia prevalence hasn´t decrease significantly. This deserves an urgent evaluation of dyslipidaemia aetiology and the adequate treatment for each case for a more personalised medicine and best patient prognosis. This could contribute to a decrease in cardiovascular mortality and morbidity.

STUDY CONTRIBUTIONS TO PUBLIC HEALTH

The characterisation of dyslipidaemia patterns based on the newly determined percentiles was the major contribution of this work for public health. The analysis performed highlights the determination of the prevalence for each lipid biomarkers that should be taken in consideration when developing new health politics. The determination of the atherosclerotic risk based in the measurement of the most atherogenic particles, never performed in our population, is also important to take in consideration when developing preventive strategies for CVD. Also it was shown the importance of the determination of these most recent biomarkers and that it can improve patient management and consequently patient prognosis. A special attention must be drawn to dyslipidaemia in women that was shown to be under diagnosed.

This study fills a gap of more than ten years without data on dyslipidaemia prevalence determined by an independent population study. The identification of a public health problem is the first step to initiate preventive measures.

6. STUDY LIMITATIONS

Using stratified random sampling techniques to estimate the prevalence of rare characteristics could imply bias, so random error should be considered in cases with small sample size. Thus, for monogenic dyslipidaemia and for the number of individuals to be above the $P90^{th}$ prior to medication for TC, LDL-C and for apoB who reached values below the $P50^{th}$, we have only mentioned the number of individuals in our sample that presented the characteristics of interest. Also, the reduction in the TC, LDL-C and apoB that we accounted for those undergoing lipid-lowering therapy might imperfectly estimate the untreated values due to the heterogeneity in drug response, dosing and variability in baseline lipid values. However, the 30% reduction in the LDL-C and 20% in the TC was implemented in a previous studies (Baigent et al., 2005; Peloso et al., 2014; Khera et al., 2016).

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8. SUPPLEMENTARY DATA

8.1. Supplementary Material and Methods

8.1.1. Study type

The e_COR Study (study of the prevalence of cardiovascular risk factors of the Portuguese population) was designed as an observational cross-sectional epidemiological study.

The study was previously approved by the National Commission for Data Protection and the National Institute of Health (INSA) Ethical Committee.

8.1.2. Sample definition

The aim was to have a stratified proportional sample with representation of genders, all Portuguese continental regions and ages pre-defined to be ≥18 years and <80 years. The database used for the sample was the National Register of users (RNU) (National Register of Users, 2017) in 2011 – individuals aged ≥18 years and <80 years. It was defined a sample with 1,685 individuals, distributed equally by the five continental regions (NUTS II), based on the following assumptions:

The determined minimum sample size to obtain results with a national representation for the determination of the prevalence of cardiovascular risk factors in the Portuguese population was 1,040 individuals, based on population data from the *Instituto Nacional de Estatística* (INE) (Instituto Nacional de Estatistica, Censos 2011), and taking into account the prevalence of hypertension (HT) determined by the study "Prevalence, knowledge, treatment and Control of Hypertension in Portugal "(PAP Study) (Sociedade Portuguesa de Cardiologia et al. 2017), which was 42% and a sampling error of 3%.

A random sampling method that involved three levels: 1) simple random selection of two health centres groups (ACES) (Cuidados de Saúde Primários, 2017) for each of the five continental health regions; 2) simple random selection of two health centres (CS) for each ACES; 3) simple random selection of participants registered in each chosen CS, weighted by the proportional size of users of each CS within the ACES and divided equally by the three defined age groups. The data of users of each CS was kindly provided by the Central Administration of the Health System (ACSS) (ACSS, 2017) in 2011.

The defined exclusion criteria were pregnant or postpartum women up to 3 months; inability to understand/speak Portuguese; declared mental disease; residence outside the

study region; telephone contact failure after 3 different attempts at different days and hours.

A total of 1,688 unrelated adults, 848 men and 840 women aged between 18 and 79, and recruited from Norte, Centro, Lisboa, Alentejo and Algarve regions were included in the e_COR Study. The rate of respondents who entered the e_COR Study was 34%, being the remaining 76% not interested in taking part in our survey, the majority due to lack of time/availability.

8.1.3. Recruitment of participants

Each selected individual was mailed an invitation letter providing information about the study. Later (1-2 weeks), one telephone contact was made to clarify any doubts about the study, verifying the inclusion criteria and pre-defined exclusion criteria. If the participant agreed, the data and sample collection was scheduled. Whenever possible the data and sample collection, took place at the INSA (Lisbon and Porto) or at the CS of the participant's residence area. When this was not possible, the field work was carried out on a private clinic in the participant's residence area.

8.1.4. Field work team

The team in each filed post consisted of a nurse (physical examination), a technician (blood collection), two psychologists (questionnaire application) and a researcher (field coordinator). When it was not possible to recruit a technician or psychologists, nurses were recruited for carrying out these tasks. All elements for this team, except the field coordinator that belonged to the research team, were subcontracted for this purpose.

8.1.5. Data collection

The data and sample collection of each participant was processed sequentially (with the signature of the informed consent always being the initial step), and consisted of the following: (**1**) read and signed informed consent; (**2**) fasting venous blood sample collection for analysis of biochemical parameters; (**3**) physical examination (blood pressure, weight, height and waist circumference measurements); (**4**) questioning based on the study questionnaire formulated by a team member. Detailed description of the 4 study steps are described below.

Step 1 – Inform consent: all participants were properly informed about the study, and had the opportunity to discuss all matters considered relevant on it, before starting their

participation. After clarification, all subjects signed informed consent to accept their participation in the study;

Step 2 – Blood collection: the blood sample was obtained after fasting for about 12 hours. To each participant approximately 16 mL of blood was withdrew for the determination of biochemical parameters, as well as for DNA extraction (1 serum gel tube 7.5 mL, 3 EDTA tubes 2.7 ml);

Step 3 – Physical examination: the physical examination consisted of measurement of blood pressure, systolic (SBP) and diastolic (DBP), weight, and height and waist circumference. The blood pressure measurement was performed in the sitting position after at least 10 minutes rest with a digital sphygmomanometer (M6 Comfort, OMRON), and a measurement was performed on the left arm and two in the right arm. The value used in the data analysis was the arithmetic average of these three measurements. Physical examination also included the determination of weight and height (digital scale SEC-899 and SEC-217 stadiometer, CEAS), with the participant using only light cloths and no shoes. However, for calculating the body mass index (BMI) 0.5 kg was removed. The measurement of waist circumference was held at the midpoint between the lower edge of the last rib and the iliac crest (flexible tape SEC-201, SECA), with the participant standing, wherever possible;

Step 4 – Questionnaire application: the questionnaire was developed by the study team and was divided into 10 main sections: personal data; recent clinical information; medication; information on high cholesterol, high triglycerides (TG); information on diabetes and HT; chronic diseases; smoking habits; eating habits; physical activity. The form consists of questions with open and closed response. The women were also asked about the use of birth control pills and/or hormone therapy, number of pregnancies, number of miscarriages and age of menopause.

8.1.6. Sample Processing

After collection, the blood was maintained at rest between 30 minutes and 3 hours, and then centrifuged at 3,000 rpm for 15 minutes. After centrifugation, the serum and plasma were stored in a fridge with controlled temperature between 2 °C and 8 °C and was then transported in a refrigerated environment to INSA where the samples were processed within a period of 36 hours. All biochemical determinations were performed at the Diagnosis and Reference Laboratory Unit (UDR) of INSA, in Lisbon or Porto.

Biomarkers for hepatic function, namely aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transpeptidase (GGT), as well as

biomarkers for the metabolism of glucose were determined for 1,676 and 1,688 individuals, respectively, in a Cobas Integra 400 plus (Roche, Risch-Rotkreuz, Switzerland) by enzymatic colorimetric and immunoturbidimetric methods, using the hexokinase enzyme. The biochemical tests for total cholesterol (TC), direct low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), TG, apolipoprotein A1 (apoA1), and apolipoprotein B (apoB) were determined for all 1,688 samples in an autoanalyser Cobas Integra 400 plus (Roche, Risch-Rotkreuz, Switzerland), also by an enzymatic colorimetric and immunoturbidimetric method. Serum levels of small, dense low-density lipoprotein cholesterol (sdLDL-C) were measured in 1,669 samples (98.87%) by direct quantification in an autoanalyser RX Daytona (Randox Laboratories, Crumlin, United Kingdom) by an enzymatic colorimetric method (sLDL-EX "Seiken").

8.1.7. Definition of study variables considered in the present study

The variables of the e_COR Study that were considered in/for the data analysis of the present study (described below) were defined based on internationally accepted criteria, and mostly criteria adapted from the World Health Organization (WHO) and scientific societies as the European Atherosclerosis Society (EAS), the European Society of Cardiology (ESC) and European Society of Hypertension (ESH).

8.1.7.1. Alcohol intake

Alcohol intake was estimated from the self-reported number of times per day, that, beer, wine and distillate drinks were consumed. It was assumed that a "standard" unit of alcohol (1 glass of 125 mL of wine, 1 bottle of 33 cL of beer, 1 glass of 40mL of distillate drink) equivalent to 10 g of absolute alcohol. According to recommendations of the European guidelines, if no contraindications to alcohol consumption are present (e.g. TG not elevated), moderate consumption – up to 20 g/day (2 units) for men and 10 g/day (1 unit) for women – is acceptable for those who drink alcoholic beverages (Perk et al., 2012; Catapano et al., 2016).

8.1.7.2. Body mass index

BMI was calculated as weight in kilograms divided by squared height in metres (kg/m^2) and further classified into normal weight, overweight and obesity according to the European guidelines (Perk et al. 2012; Catapano et al., 2016).

8.1.7.3. Diabetes

It was considered that an individual had diabetes when the level of fasting glucose was greater than 126 mg/dL (determined on 2 separate occasions) or below that threshold, when under therapy for diabetes; presenting pre-diabetes when the glucose level was between 110 mg/dL and 126 mg/dL (ACSS, 2017; Cuidados de Saúde Primários, 2017).

Recall that the test for glucose tolerance was not determined, so the results are presented only on the basis of fasting glucose values and information of patients on diabetic medication.

8.1.7.4. Family history of premature cardiovascular disease

According to the ESC/EAS (Perk et al., 2012; Catapano et al., 2016), the pCVD antecedent risk factor is related to the existence of one or more family members of first degree with premature CVD. The CVD was defined if any of the following events: angina, myocardial infarction, percutaneous transluminal coronary angioplasty, or coronary artery bypass grafting. CVD was defined as premature when appear before age 55 in men and before age 65 in women. While, in many cases, the participant does not remember the age of the relative when the event occurred, the information on relative age at the study date was taken in consideration and if within the considered prematurity limits it was admitted as pCVD history (De Sutter et al., 2003; Perk et al., 2012; Catapano et al., 2016).

8.1.7.5. Fruit and vegetable consumption

According to WHO WHO, 2000; Perk et al., 2012; Catapano et al., 2016) and the ESC/EAS each individual should eat at least 400 g of fruit and vegetables daily, corresponding to 5 servings of these foods, which is the recommendation for preventing cardiovascular disease. This consumption was established as reference.

8.1.7.6. Hormone users

Women were classified as hormone users if at the time blood collection they said they were taking birth control pills or under any hormone therapy.

8.1.7.7. Hypertension

An individual was considered to have prehypertension if presented SBP ≥ 130 < 140 mmHg and/or DBP ≥ 85 < 90 mmHg, and was considered to have HT if

SBP ≥140 mmHg and/or DBP ≥ 90 mmHg or below these values if under anti-hypertensive therapy (Mancia et al., 2013).

8.1.7.8. Hypothyroidism/hyperthyroidism

It is considered that an individual had hypothyroidism or hyperthyroidism if they said they were diagnosed or if they were or under treatment for hypothyroidism or hyperthyroidism.

8.1.7.9. Lipid-lowering therapy

Individuals reporting any lipid-lowering therapy for cholesterol and/or triglycerides at the time blood collection were considered under lipid-lowering therapy.

8.1.7.10. Overweight/obesity

It is considered that the individual is overweight when BMI is between 25 and 29.9 kg/m^2 and obese when above than 30 kg/m^2 (WHO, 2000; Perk et al., 2012; Catapano et al., 2016).

8.1.7.11. Physical activity

In the analysis of physical activity, it was considered the criteria set by the International Physical Activity Questionnaire (IPAQ), which reflects different levels of physical activity, low, moderate and vigorous intensity, in different contexts, including: professional, domestic (including gardening), transport and leisure (sports and recreation). So individuals were classified into three levels of physical activity: high, moderate or low (Hagströmer, Oja, and Sjöström, 2006; Lee et al., 2011).

8.1.7.12. Smoking status

Individuals were classified as smoker when reporting any cigarette smoke daily/occasionally at the time blood collection.

8.1.8. Molecular analysis

8.1.8.1. Isolation of genomic DNA from blood

Genomic DNA was isolated from peripheral blood EDTA samples, by the salting method, using an adaptation of the protocol described by Lahore et al. (1991) (Lahiri et al., 1991). DNA integrity was assessed by agarose gel electrophoresis (1% agarose gel), and DNA purity (provided by the A260/A280 and A260/A230 ratios) and concentration were determined using the NanoDrop™ 1000 Spectrophotometer (Termo

Fisher Scientific, Waltham, Massachusetts, EUA). For targeted next-generation sequencing (NGS), DNA concentration was more-precisely assessed using the Qubit™ dsDNA fluorometric quantitation assay (Termo Fisher Scientific, Waltham, Massachusetts, EUA) according to the manufacturer's instructions (Life Technologies., 2010).

8.1.8.2. Targeted sequencing

The NGS libraries were prepared using the SureSelect^{QXT} Target Enrichment for Illumina Multiplex Sequencing (Agilent Technologies, 2016) (Agilent Technologies, Santa Clara, CA, USA). DNA library quantity and quality was assessed using an Agilent TapeStation system and D1000 ScreenTape according to the manufacturer's instructions (Agilent Technologies, 2017). Hybridisation and capture genomic DNA (gDNA) libraries were performed using the SureSelect^{QXT} 1 kb–499 kb Custom Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions(Agilent Technologies, 2016). Indexed library DNA quantity and quality was assessed using an Agilent TapeStation system and High Sensitivity D1000 ScreenTape according to the manufacturer's instructions (Agilent Technologies, 2017). For a more-precisely quantification of the Target enriched samples prior to pooling, we used the Qubit™ dsDNA high sensitivity fluorometric quantitation assay (Termo Fisher Scientific, Waltham, Massachusetts, EUA) according to the manufacturer's instructions (Life Technologies., 2010). Samples that failed in any step of the solution hybrid selection component of the targeted sequencing process were excluded. The resulting gDNA libraries were paired-end sequenced (2 x 75 bp) in Illumina MiSeq equipment (Illumina), according to the manufacturer's instructions (performed by the Technology and Innovation Unit, INSA). Of targeted regions, 97% were covered at ≥ Q30. The generated FASTQ files were aligned to the human genome reference GRCh37 (hg19) and scrutinized using SureCall data analysis software (version 3.0; Agilent Technologies, Santa Clara, CA, USA) (Agilent Technologies, 2015). The median read depth was 239.00 (IQR 192.00) (ranging from 10 to 951).

8.1.8.2.1. Targeted sequencing data analysis

Data analysis procedure was simplified in Supplementary Figure 1. All data of the 26 genes panel were analysed for quality and variant analysis: 1) confirmation of correct reference sequence; 2) exclusion of variants with an allele frequency below 30%; 3) exclusion of downstream variants 100 base pairs (bp) after stop codon, or intronic variants with more than 10 bp before or after exons. Following these, we first looked for rare variants with a minor allele frequency (MAF) below 3% (particularly defined for our analysis) by the following order, missense, intronic, 5 prime UTR (5'UTR) and 3 prime

UTR (3'UTR), and silent. Then, we looked for missense, intronic, and 5'UTR and 3' UTR common variants with MAF above 3%, respectively. Lastly, we looked for small deletions and insertions (indels) by only including variants with a frequency below 30% in our sample. All unknown potential variants were investigated by research in literature and *in silico* programs (when applicable), Protein Variation Effect Analyzer (PROVEAN) (Choi and Chan, 2015). Sorting Tolerant From Intolerant (SIFT) (Ng and Henikoff, 2003), PolyPhen-2 (Adzhubei et al., 2013), Consensus Deleteriousness score of missense SNVs (Condel) (González-Pérez and López-Bigas, 2011), and MutationTaster(Schwarz et al., 2014) for prediction of protein structure/function changes and evolutionary conservation; Grantham score (Grantham, 1974) and PhyloP (Siepel et al., 2006) for amino acid/nucleotide conservation analysis; and Human Splicing Finder (Desmet et al., 2009), Neural Network Splice Site Prediction Tool (NNSSP) (Reese et al., 1997), and Neural Network Predictions of Splice Sites in Human (NetGen2) (Hebsgaard et al., 1996) for prediction of splicing defects. For missense common variants (MAF above 3%), we have only considered those with evidence of association with the phenotypes, and with a frequency below 30% in our sample (excepted in cases where more than 70% fall within the phenotype). Homozygous and heterozygous were confirmed by Sanger sequencing.

8.1.9. Stratified random sampling techniques

The stratified random sampling techniques allowed us to build a weighed estimator of the mean values and their correspondent 95% confidence intervals (CI), standard deviation (SD), variance, and standard error (SE), as well as of the prevalence (expressed as percentage) and their correspondent 95% CI, with known asymptotic probabilistic behaviour leading to the calculation of these estimations (Supplementary Tables 2-4). For a better understanding of such estimation, an example will be provided.

To calculate the mean value of LDL-C for overall population, for example, this population was divided by region, then regions were divided by genders, and age group then divided genders. Stratum weights were calculated in each region, gender and age group, according to the demographic composition of the adult population resident in Portugal. The mean values, variance, SD, and SE of each age group, in each gender of each region, were calculated directly using R (version 3.1.2) software (R: The R Project for Statistical Computing, 2017). The mean values and variance calculated in R, together with stratum weights previously calculated, were then used to estimate the mean values and variance of each gender. Following this, these mean values and variance estimated of each gender, together with stratum weights determined previously, were then used to estimate the mean values and variance of each region. Finally, these mean values and

variance estimated for each region, together with stratum weights determined previously, were used to estimate the mean value and variance for a total population.

The same strategy was used to estimate the prevalence, although considering stratified sampling rules for proportions. Estimators used for this analysis are described below.

8.2. Supplementary Tables

8.2.1. Supplementary Table 1

Supplementary Table 1 – Description of the notations used in the equations.

Notation	Description
$\boldsymbol{N}$	Total number of units according to the real population
$\boldsymbol{N_h}$	Total number of units in a specific strata according to the real population
$\boldsymbol{n_h}$	Number of units in sample
$\boldsymbol{W_h}$	Stratum weight
$\boldsymbol{\bar{y}_h}$	Sample mean[a]
$\boldsymbol{\bar{y}_{st}}$	Stratified sample mean
$\boldsymbol{V(\bar{y}_{st})}$	Variance of stratified sample mean
$\boldsymbol{S_h^2}$	Sample variance[a]
$\boldsymbol{S_{st}^2}$	Stratified sample variance
$\boldsymbol{SE_{\bar{y}_{st}}}$	Standard error of stratum mean
$\boldsymbol{Lower\ limit_{\bar{y}_{st}}}$	Lower limit of the 95% CI of stratified sample mean
$\boldsymbol{Upper\ limit_{\bar{y}_{st}}}$	Upper limit of the 95% CI of stratified sample mean
$\boldsymbol{a_h}$	Number of units of interest in sample
$\boldsymbol{\hat{p}_h}$	Sample proportion
$\boldsymbol{\hat{p}_{st}}$	Stratified sample proportion
$\boldsymbol{V(\hat{p}_{st})}$	Variance of stratified sample proportion
$\boldsymbol{SE_{\hat{p}_{st}}}$	Standard error of stratified sample proportion
$\boldsymbol{Lower\ limit_{\hat{p}_h}}$	Lower limit of the 95% CI of sample proportion
$\boldsymbol{Upper\ limit_{\hat{p}_h}}$	Upper limit of the 95% CI of sample proportion
$\boldsymbol{Lower\ limit_{\hat{p}_{st}}}$	Lower limit of the 95% CI of stratified sample proportion
$\boldsymbol{Upper\ limit_{\hat{p}_{st}}}$	Upper limit of the 95% CI of stratified sample proportion

h, stratum *h*; *st*, stratified; ***i***, unit within the stratum; ***L***, number of values; CI, confidence interval; SE, standard error.

[a]Sample mean and sample variance was calculated directly using R packages.

8.2.2. Supplementary Table 2

Supplementary Table 2 – Equations used to build a weighed estimator of the mean values and their correspondent 95% confidence intervals, standard deviation, variance, and standard error.

Equation	Description
$\boldsymbol{W_h = \frac{N_h}{N}}$	Calculate the stratum weight
$\boldsymbol{\bar{y}_{st} = \sum_{h=i}^{L} W_h \bar{y}_h}$	Estimate for stratified sample mean
$\boldsymbol{S_{st}^2 = \sum_{h=i}^{L} W_h S_h^2 + \sum_{h=i}^{L} W_h (\bar{y}_{st} - \bar{y}_h)^2}$	Estimate for stratified sample variance
$\boldsymbol{V(\bar{y}_{st}) = \sum_{h=i}^{L} W_h^2 V(\bar{y}_h)}$	Estimate for variance of stratified sample mean
$\boldsymbol{SE_{\bar{y}_{st}} = \sqrt{V(\bar{y}_{st})}}$	Calculate the standard error of stratified sample mean
$\boldsymbol{Lower\ limit_{\bar{y}_{st}} = \bar{y}_{st} - 1.96\sqrt{V(\bar{y}_{st})}}$	Estimate for lower limit of the 95% CI of stratified sample mean
$\boldsymbol{Upper\ limit_{\bar{y}_{st}} = \bar{y}_{st} + 1.96\sqrt{V(\bar{y}_{st})}}$	Estimate for upper limit of the 95% CI of stratified sample mean

h, stratum *h*; *st*, stratified; ***i***, unit within the stratum; ***L***, number of values; CI, confidence interval; SE, standard error.

8.2.3. Supplementary Table 3

Supplementary Table 3 – Equations used to build a weighed estimator of the prevalence (expressed as percentage), their correspondent 95% confidence intervals, standard deviation, variance, and standard error.

Equation	Description
$\widehat{\boldsymbol{p}}_{\boldsymbol{h}} = \frac{\boldsymbol{a_h}}{\boldsymbol{n_h}}$	Calculate the sample prevalence
$\widehat{\boldsymbol{p}}_{\boldsymbol{st}} = \sum_{\boldsymbol{h=i}}^{\boldsymbol{L}} \widehat{\boldsymbol{p}}_{\boldsymbol{h}} \boldsymbol{W_h}$	Estimate for stratified sample prevalence
$\boldsymbol{V}(\widehat{\boldsymbol{p}}_{\boldsymbol{st}}) = \sum_{\boldsymbol{h=i}}^{\boldsymbol{L}} \boldsymbol{W_h^2 V}(\widehat{\boldsymbol{p}}_{\boldsymbol{h}})$	Estimate for variance of stratified sample prevalence
$\boldsymbol{SE}_{\widehat{\boldsymbol{p}}_{\boldsymbol{st}}} = \sqrt{\boldsymbol{V}(\widehat{\boldsymbol{p}}_{\boldsymbol{st}})}$	Calculate the standard error of stratified sample prevalence
$\boldsymbol{f_h} = \frac{\boldsymbol{n_h}}{\boldsymbol{N_h}}$	Calculate the sampling fraction in the stratum[a]
$\boldsymbol{Lower\ limit}_{\widehat{\boldsymbol{p}}_{\boldsymbol{h}}} = \widehat{\boldsymbol{p}}_{\boldsymbol{h}} - \boldsymbol{1.96}\sqrt{\boldsymbol{(1-f_h)V}(\widehat{\boldsymbol{p}}_{\boldsymbol{h}})} + \frac{\boldsymbol{1}}{\boldsymbol{2n_h}}$	Estimate for lower limit of the 95% CI of sample prevalence
$\boldsymbol{Upper\ limit}_{\widehat{\boldsymbol{p}}_{\boldsymbol{h}}} = \widehat{\boldsymbol{p}}_{\boldsymbol{h}} + \boldsymbol{1.96}\sqrt{\boldsymbol{(1-f_h)V}(\widehat{\boldsymbol{p}}_{\boldsymbol{h}})} + \frac{\boldsymbol{1}}{\boldsymbol{2n_h}}$	Estimate for upper limit of the 95% CI of sample prevalence
$\boldsymbol{Lower\ limit}_{\widehat{\boldsymbol{p}}_{\boldsymbol{st}}} = \widehat{\boldsymbol{p}}_{\boldsymbol{st}} - \boldsymbol{1.96}\sqrt{\boldsymbol{V}(\widehat{\boldsymbol{p}}_{\boldsymbol{st}})}$	Estimate for lower limit of the 95% CI of stratified sample prevalence
$\boldsymbol{Upper\ limit}_{\widehat{\boldsymbol{p}}_{\boldsymbol{st}}} = \widehat{\boldsymbol{p}}_{\boldsymbol{st}} + \boldsymbol{1.96}\sqrt{\boldsymbol{V}(\widehat{\boldsymbol{p}}_{\boldsymbol{h}})}$	Estimate for upper limit of the 95% CI of stratified sample prevalence

h, stratum *h*; *st*, stratified; ***i***, unit within the stratum; ***L***, number of values; CI, confidence interval; SE, standard error.

[a]When f_h is approximately equal to zero, f_h can be considered negligible.

8.2.4. Supplementary Table 4

Supplementary Table 4 – Baseline characteristics of all the individuals from the e_COR Study.

Variable	Total	Range	Men	Range	Women	Range
N	1,688	..	848	..	840	..
Age, years (SD)[a]	50.17 (18.32)	18-79	51.02 (18.29)	18-79	49.31 (18.31)	18-79
BMI (n=1,687), kg/m^2 (SD)[a]	26.88 (4.87)	16-56	27.17 (4.33)	16-45	26.59 (5.35)	16-56
Overweight/obesity, n (%)[c]	1070 (63.39)	..	582 (68.63)	..	488 (58.10)	..
Physical inactivity, n (%)[d]	496 (29.38)	..	260 (30.66)	..	236 (28.10)	..
Sedentarism, n (%)	448 (26.54)	..	228 (26.89)	..	220 (26.19)	..
Physical inactivity and sedentarism, n (%)[d]	198 (11.73)	..	97 (11.44)	..	87 (10.36)	..
Balanced diet, n (%)[c]	1199 (71.03)	..	646 (76.18)	..	553 (65.83)	..
Smokers, n (%)	375 (22.22)	..	231 (27.24)	..	144 (17.14)	..
Alcohol intake, g/L[a]	9.61 (15.58)	0-160	16.14 (18.73)	0-160	3.02 (6.88)	0-104
SBP, mmHg (SD)[a]	128.28 (22.42)	79-235	134.15 (20.50)	87-235	122.32 (22.72)	79-208
DBP, mmHg (SD)[a]	79.91 (11.40)	47-133	81.62 (11.13)	51-133	78.18 (11.42)	47-129
Prehypertension, n (%)[c]	116 (6.87)	..	78 (9.20)	..	38 (4.52)	..
Hypertension, n (%)[c]	819 (48.52)	..	443 (52.24)	..	376 (44.76)	..
Diabetes, n (%)[c]	198 (11.73)	..	130 (15.33)	..	68 (8.10)	..
Hyperthyroidism/hypothyroidism, n (%)	53 (3.14)	..	9 (1.06)	..	44 (5.24)	..
Cardiovascular disease, n (%)	76 (4.50)	..	51 (6.01)	..	25 (2.98)	..
Family history of pCVD[c] or hypercholesterolaemia, n (%)	449 (26.60)	..	180 (21.23)	..	269 (32.02)	..
Receiving statins, n (%)	454 (26.90)	..	228 (26.89)	..	226 (26.90)	..
ALT (n=1,675), mg/dL (IQR)[b]	17.00 (13-25)	4-228	22.00 (16-31)	5-228	15.00 (12-19)	4-180
AST (n=1,673), mg/dL (IQR)[b]	19.00 (16-23)	7-233	21.00 (18-25)	10-112	17.00 (15-21)	7-233
GGT (n=1,678), mg/dL (IQR)[b]	17.00 (12-28)	2-481	23.00 (16-38)	5-481	13.00 (9-18)	2-332
Total cholesterol, mg/dL (IQR)[b]	192.00 (168-216)	87-417	191.00 (165-218)	87-338	192.00 (171-215)	94-417

Continuation of Supplementary Table 4 – Baseline characteristics of all the individuals from the e_COR Study.

Variable	Total	Range	Men	Range	Women	Range
LDL-C, mg/dL (IQR)[b]	117.00 (95-139)	31-296	122.00 (97-144)	31-256	113.00 (93-135)	31-296
HDL-C, mg/dL (IQR)[b]	54.00 (45-65)	22-135	49.00 (41-58)	23-112	61.00 (52-71)	22-135
Triglycerides, mg/dL (IQR)[b]	94.00 (79-129)	21-517	102.00 (72-143)	27-517	86.00 (66-120)	21-461
ApoB (n=1.686), mg/dL (IQR)[b]	91.00 (76-107)	11-193	95.00 (78-112)	11-184	88.00 (74-103)	21-193
ApoA1, mg/dL (IQR)[b]	152.00 (134-171)	78-280	143.00 (127-159)	78-252	162.50 (144-182)	84-280
sdLDL-C (n=1,667), mg/dL (IQR)[b]	27.00 (19-36)	5-123	29.00 (21-40)	5-123	24.00 (19-32)	4-91
Non-HDL-C, mg/dL (IQR)[b]	135.00 (110-160)	44-326	140.00 (113-168)	44-290	131.00 (108-154)	49-326
ApoB/apoA1 ratio (n=1,686), mg/dL (IQR)[b]	0.59 (0.47-0.75)	0.1-1.6	0.66 (0.52-0.81)	0.1-1.6	0.54 (0.22-0.44)	0.2-1.5
sdLDL-C/LDL-C ratio (n=1,667), mg/dL (IQR)[b]	0.23 (0.19-0.28)	0.1-1.0	0.24 (0.20-0.30)	0.1-1.0	0.22 (0.18-0.26)	0.1-0.6
Lp(a) (n=895), mg/dL (IQR)[b]	11.50 (6-28)	3.4-178	11.80 (6-23)	3.4-137	11.30 (6-22)	3.4-178

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; pCVD, premature cardiovascular disease; ALT, alanine transaminase; AST, Aspartate transaminase; GGT, gamma-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; sdLDL-C, small, dense low-density lipoprotein; Non-HDL-C, non-high-density lipoprotein cholesterol; SD, standard deviation; IQR, interquartile range.

Data are presented as n (%), mean (SD)[a] or median (IQR)[b].

[c]Overweight/obesity, balanced diet, prehypertension/hypertension, diabetes, and pCVD were classified according to the national and/or international guidelines and recommendations, as described previously in this section.

[d]Physical inactivity was defined according to IPAQ (Hagströmer, Oja, and Sjöström, 2006; Lee et al., 2011), and sedentarism was defined as more than 6 hours sitting per day.

8.2.5. Supplementary Table 5

Supplementary Table 5 – List of dyslipidaemia-associated genes included in the targeted next-generation sequencing.

Gene	Protein	Chr	Start	Stop	ID	Reference	Phenotype
MTTP	Microsomal triglyceride transfer protein	4	100485240	100545154	ENSG00000138823	NM_000253	Abetalipoproteinaemia
SAR1B	Secretion associated Ras related GTPase 1B	5	133936839	133968533	ENSG00000152700	NM_001033503	Anderson disease
STAP1	Signal-transducing adaptor protein 1	4	68424446	68473055	ENSG00000035720	NM_012108	Body Weight; HDL-C; CHD; Stroke
LIPA	Lysosomal acid lipase/cholesteryl ester hydrolase	10	90973326	91011660	ENSG00000107798	NM_001127605	Cholesteryl Ester Storage Disease; Wolman syndrome
ANGPTL3	Angiopoietin-like 3	1	63063158	63071976	ENSG00000132855	NM_014495	Familial combined hypolipidemia
LDLRAP1	Low density lipoprotein receptor adaptor protein	1	25870071	25895377	ENSG00000157978	NM_015627	Familial hypercholesterolaemia
PCSK9	Proprotein convertase subtilisin/kexin type 9	1	55505149	55530526	ENSG00000169174	NM_174936	Familial hypercholesterolaemia
LDLR	Low density lipoprotein receptor protein	19	11200037	11244506	ENSG00000130164	NM_000527	Familial hypercholesterolaemia
APOB	Apolipoprotein B	2	21224301	21266945	ENSG00000084674	NM_000384	Familial hypercholesterolaemia; Hypobetalipoproteinemia
LIPC	Lipase C	15	58702953	58861073	ENSG00000166035	NM_000236	Hepatic lipase deficiency
SCARB1	Scavenger receptor class B member 1	12	125262174	125348519	ENSG00000073060	NM_005505	High HDL-C
CETP	Cholesteryl ester transfer protein	16	56995835	57017757	ENSG00000087237	NM_000078	High HDL-C
FLT1	Fms related tyrosine kinase 1	13	28874481	29069265	ENSG00000102755	NM_002019	High LDL-C
LPL	Lipoprotein lipase	8	19796582	19824770	ENSG00000175445	NM_000237	Hypertriglyceridaemia
GPIHBP1	Glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1	8	144295068	144299044	ENSG00000277494	NM_178172	Hypertriglyceridaemia
APOA5	Apolipoprotein A5	11	116660086	116663136	ENSG00000110243	NM_052968	Hypertriglyceridaemia
APOC3	Apolipoprotein C3	11	116700624	116703787	ENSG00000110245	NM_000040	Hypertriglyceridaemia
GPD1	Glycerol-3-phosphate dehydrogenase 1	12	50497602	50505103	ENSG00000167588	NM_005276	Hypertriglyceridaemia
LMF1	Lipase maturation factor 1	16	903634	1031318	ENSG00000103227	NM_022773	Hypertriglyceridaemia
APOE	Apolipoprotein E	19	45409039	45412650	ENSG00000130203	NM_000041	Hypertriglyceridaemia
APOC2	Apolipoprotein C2	19	45449239	45452822	ENSG00000234906	NM_000483	Hypertriglyceridaemia
ABCA1	ATP binding cassette subfamily A member 1	9	107543283	107690527	ENSG00000165029	NM_005502	Low HDL-C
APOA1	Apolipoprotein A1	11	116706467	116708338	ENSG00000118137	NM_000039	Low HDL-C
LCAT	Lecithin-cholesterol acyltransferase	16	67973787	67978656	ENSG00000213398	NM_000229	Low HDL-C

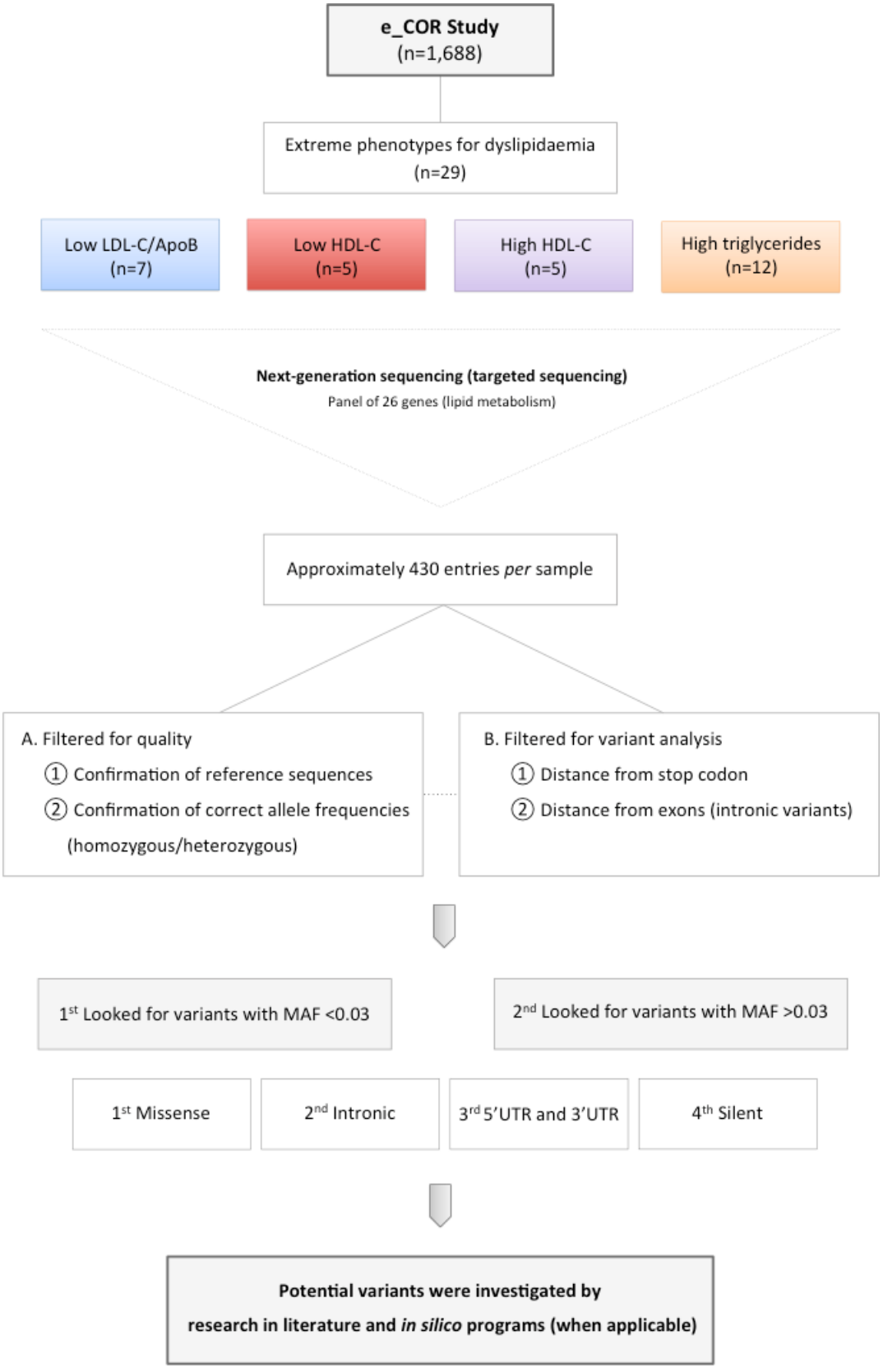
Continuation of Supplementary Table 5 – List of dyslipidaemia-associated genes included in the targeted NGS.

Gene	Protein	Chr	Start	Stop	ID	Reference	Phenotype
ABCG5	ATP binding cassette subfamily G member 5	2	44039611	44066039	ENSG00000138075	NM_022436	Sitosterolemia
ABCG8	ATP binding cassette subfamily G member 8	2	44066103	44105947	ENSG00000143921	NM_022437	Sitosterolemia

NGS, next-generation sequencing; Chr, chromosome; ID, identification; CHD; coronary heart disease; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

8.3. Supplementary Figures

8.3.1. Supplementary Figure 1



Supplementary Figure 1 – Schematic representation of the targeted next-generation sequencing data analysis. From a panel of 26 targeted genes, more than 400 results were obtained for each sample. All data were analysed for (A) quality and (B) variants investigation. (A) quality: 1) confirmation of correct reference sequence, and 2) exclusion of variants with an allele frequency below 30%. (B) variants investigation: 1) exclusion of downstream variants 100 base pairs (bp) after stop codon, or 2) intronic variants with more than 10 bp before or after exons. In a first phase we looked for rare variants with a minor allele frequency (MAF) below 3% by the following order, missense, intronic, 5 prime UTR (5'UTR) and 3 prime UTR (3'UTR), and silent. In a second phase we looked for missense, intronic, and 5'UTR and 3' UTR common variants with MAF above 3%, following this order. For missense variants with MAF above 3%, we have only considered those with evidence of association with the phenotypes, and with a frequency below 30% in our sample (excepted in cases where more than 70% fall within the phenotype). All unknown potential variants were investigated by research in literature and *in silico* programs (when applicable).

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Chapter 4

THE FH PHENOTYPE: MONOGENIC FAMILIAL HYPERCHOLESTEROLAEMIA, POLYGENIC DYSLIPIDAEMIA AND OTHER CAUSES

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ABSTRACT

Familial Hypercholesterolaemia (FH) is a monogenic disorder characterised by high low-density lipoprotein cholesterol (LDL-C) values and increased cardiovascular risk. However, in only about 40%-50% an FH-causing mutation is found. The aim of this work was to characterise the origin of FH phenotype in a cohort of patients with clinical diagnosis of FH. About 731 clinical FH patients (children and adults) have been referred to our laboratory to find the genetic cause of their phenotype. *LDLR*, *APOB*, *PCSK9*, *APOE*, *LIPA*, *LDLRAP1*, *ABCG5*/8 genes have been studied. The 6-SNP LDL-C genetic risk score (GRS) for polygenic hypercholesterolaemia has been validated in our population. Dutch and Simon Broome clinical FH criteria were also compared. A total of 38.71% of patients have FH, 13.68% polygenic hypercholesterolaemia and 0.82% have other lipid disorders, giving a total of 53.21% of patients where the cause of hypercholesterolaemia has been identified. If all variants of uncertain significance were pathogenic, the identification rate would increase to 59.23%. In the remaining FH negative patients, about 9.39% presented low LDL-C GRS and could have another cause of monogenic dyslipidaemia. Results comparing the FH clinical criteria suggested no significant different discriminative power. All known causes of the FH phenotype should be investigated. A correct identification of the origin of the dyslipidaemia is important for patient management, including the implementation of the best therapeutical measure for the best patient prognosis.

Keywords: Familial hypercholesterolaemia; monogenic dyslipidaemia; polygenic dyslipidaemia; genetic risk score; lipid disorders.

1. INTRODUCTION

Familial Hypercholesterolaemia (FH) is an autosomal dominant condition characterised by substantially raised plasma concentrations of low-density lipoprotein cholesterol (LDL-C) in plasma from birth. FH is the most common inherited lipid disorder associated to premature coronary heart disease (pCHD), with a frequency around 1:250-500 in most populations (Nordestgaard et al., 2013).

The genetic causes of FH are loss-of-function mutations, mainly in the LDL receptor gene (*LDLR*) (Stenson et al., 2014) or apolipoprotein B (*APOB*) gene (Innerarity et al.,1990; Motazacker et al., 2012; Alves et al., 2014), and gain of function mutations in the proprotein convertase subtilisin/kesin type 9 gene (*PCSK9*) (Abifadel et al., 2003). However, an increasing number of FH phenocopies are being identified and a few individuals with a clinical diagnosis have been found to have rare variants in other genes, such as apolipoprotein E (*APOE*) (Marduel et al., 2013), ATP-binding cassette sub-family G member 5/8 (*ABCG5*/*8)* (Rios et al., 2010), or lysosomal acid lipase (*LIPA*) (Hegele et al., 2015; Chora et al., 2017b). Recently studies reported that some of the clinically diagnosed cases of FH could possibly have a polygenic cause due to the inheritance of common LDL-C raising alleles, with a cumulative effect leading to an increase in LDL-C (at the level of FH range) (Talmud et al., 2013; Futema et al., 2015). Even in patients with a disease causing mutation, this polygenic contribution could also be found, reflecting a variable FH phenotype. Different scores have been suggested to estimate the polygenic contribution by a fraction of millimole per litter, although using a different combination of single nucleotide polymorphisms (SNP) (Talmud et al., 2013; Futema et al., 2015; Hegele et al., 2015). In terms of cardiovascular risk assessment, is of great importance to distinguish between a monogenic, polygenic or environmental dyslipidaemia, since the cardiovascular disease (CVD) risk has been demonstrated to be 22-fold increases in FH patients with a causative mutation compared to a 6-fold increase when only the LDL values reach the same FH levels (Khera et al., 2016).

The prevalence of genetically identified FH patients can vary across different cohorts (Benn et al., 2012; Futema et al., 2012; Motazacker et al., 2012; Bertolini et al., 2013; Nordestgaard et al., 2013) and may be due to differences in molecular diagnostic methodologies, but can also be due to differences in the clinical criteria applied. There are two mainly used criteria for clinical diagnosis of FH, the Dutch Lipid Clinic Network (DLCN) (Austin et al., 2004) criteria and the Simon Broome (SB) Register (Group, 1991) criteria, which is also endorsed by the National Institute for Clinical Health and Excellence

(NICE), and is the one used in the Portuguese FH Study. The SB criteria take into consideration cholesterol concentrations, clinical characteristics, molecular diagnosis, and family history of hypercholesterolaemia and/or pCHD and defines cut off values, while the DLCN for the same characteristics uses a point system – DLCN score (DLCNS). SB criteria defines patients in definite FH, when a mutation causing disease has been identified or the patient or first degree relative has tendon xanthoma and possible FH in the other cases; for DLCS a total point score of greater than 8 is considered definite FH, 6-8 as probable FH, and 3-5 is considered as possible FH. Only SB criteria can be applied to children.

Taking all these aspects in consideration, it is clear the importance of the correct identification of the aetiology of the dyslipidaemia in order to implement specific interventions for CVD prevention. Here, we report the characterisation of the FH phenotype in the Portuguese FH Study cohort, including monogenic, polygenic and other causes for the hypercholesterolaemic phenotype. We also validated the 6-SNP LDL-C genetic risk score (GRS) (Talmud et al., 2013; Futema et al., 2015) in the Portuguese population, and compared results using different clinical FH criteria, the DLCN and SB criteria.

2. MATERIAL AND METHODS

The Portuguese FH Study is a research project coordinated by the National Institute of Health (INSA) supported mainly by external funds and free of charge for all patients and health institutions. INSA Ethical Committee, and the National Data Protection Commission previously approved the study protocol and database. Written informed consent was obtained from all participants before their inclusion in the study.

2.1. Monogenic dyslipidaemia

2.1.1. Study population

A total of 731 index patients (311 children and 420 adults) were enrolled in the Portuguese FH Study from 1999 and 2016, referred from different clinical specialties with a clinical diagnosis of FH, according to the SB criteria, as previously described (Bourbon et al., 2006), but with a single adaptation regarding individuals aged 16-18 that were included with the SB criteria for children due to their mild phenotype. Additionally, 1777 relatives (393 children and 1384 adults) were referred to the Portuguese FH Study cascade-screening program (with or without a clinical diagnosis of FH).

2.1.2. Molecular analysis

The genetic diagnosis was performed by the molecular analysis of *LDLR* (including the study of splice regions and large rearrangements), *APOB* (two fragments of exons 26 and 29), and *PCSK9* genes, as previously reported (Medeiros et al., 2010). Selected patients, where a mutation was not found in the previously studied genes, were further investigated for other monogenic causes of dyslipidaemia; this was performed by polymerase chain reaction (PCR) and Sanger sequencing of the following genes: *APOE*, *LIPA,* LDLR adapter protein 1 (*LDLRAP1*), *ABCG5,* and *ABCG8*. In all cases sequences were analysed with Staden software (Bonfield et al., 1995; Chora et al., 2017b) and the references used were NM_000527 for *LDLR*, NM_000384 for *APOB*, NM_174936 for *PCSK9*, NM_000041 for *APOE*, NM_015627 for *LDLRAP1*, NM_022436 for *ABCG5*, and NM_022437 for *ABCG8*. Complementary DNA (cDNA) numbering was considered according to the Human Genome Variation Society (HGVS) nomenclature (den Dunnen and Antonarakis, 2000). All variants were checked with Mutalyzer 2.0, as recommended by HGVS. Variants were classified as pathogenic, likely pathogenic, benign, likely benign, or variant of unknown significance (VUS), according to Chora et al. (Chora et al., 2017a) The variants reported in the present study were considered “novel” if they were not described before, and “novel PT” if they were found for the first time in Portugal, but have been previously reported in another country.

2.1.3. In silico analysis

All rare variants not previously described were investigated by literature screening and *in silico* programs (when applicable). *In silico* analysis was performed as described before (Medeiros et al., 2016).

2.2. Polygenic hypercholesterolaemia

2.2.1. Study populations

For the polygenic hypercholesterolaemia study, the e_COR Study population (Bourbon et al., 2018) was used as reference group for the LDL-C GRS analysis. The score was then applied to the Portuguese FH Study population.

2.2.2. LDL-C genetic risk score analysis

A total of 1,563 genomic DNA samples from the e_COR Study (men and women) and 455 index cases from the Portuguese FH Study with an identified mutation (94 children and 118 adults) and without an identified mutation (92 children and 151 adults) were sent

to aScidea Computational Biology Solutions Company (Barcelona, Spain) to be genotyped for a set of 6 SNPs, using the OpenArray™ technology (Applied BioSystems., 2011) (Life Technologies, Carlsbad, California, US). The 6 SNPs were selected from the LDL-C GRS, previously reported in the characterisation of polygenic hypercholesterolaemia, namely cadherin EGF LAG seven-pass G-type receptor 2 (*CELSR2*)/sortilin 1 (*SORT1*) (rs629301), *APOB* (rs1367117), *ABCG5/8* (rs4299376), *LDLR* (rs6511720) and *APOE* (rs7412 and rs429358) (Futema et al., 2015). The samples that did not meet the quality criteria required for the procedure were not genotyped and in a few samples some SNPs could not be genotyped. A database with all genotyping results was constructed in-house for further analysis, and all variables were created according to the statistical analysis approach.

2.2.2.1. LDL-C genetic risk score calculation

The LDL-C GRS based on 6 LDL-C lead SNPs was calculated for each individual, using the weighted sum of the effect sizes of the risk allele (Supplementary Table 1), as previously described (Talmud et al., 2013; Futema et al., 2015). The effect sizes were the beta coefficients for per-allele change in LDL-C, reported by the Global Lipids Genetics Consortium (GLGC) (Teslovich et al., 2010). The *APOE* weights were based on haplotypic effects. All weights were taken from Talmud et al. (2013) and Futema et al. (2015) studies (Talmud et al., 2013; Futema et al., 2015). The e_COR population was used as control to determine the LDL-C GRS values and their corresponding LDL-C for the Portuguese population, to verify if plasma LDL-C could be due to the influence of a combination of those 6 LDL-C SNPs. Individuals with selected characteristics known to affect lipid metabolism, such as medical history of diabetes, hyperthyroidism and hypothyroidism, and also individuals with missing alleles, were excluded from this analysis, leaving 1318 individuals. Calculated LDL-C GRS values from e_COR population were distributed in percentiles to further analyse how far patients with a clinical diagnosis of FH have higher LDL-C GRS than individuals from the general population, and the 75th percentile (P75th) LDL-C GRS value was chosen as the cut-off for a polygenic hypercholesterolaemia.

2.2.3. Correlations among lipid biomarkers and the LDL-C genetic score values

Pearson correlation was used to evaluate associations between total cholesterol (TC), LDL-C, high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), apolipoprotein B (apoB), apolipoprotein A1 (apoA1), non-HDL-C, and small dense LDL-C cholesterol (sdLDL-C) biochemical values from e_COR population, and the LDL-C GRS

values. Non-HDL-C values were calculated as previously described: TC minus HDL-C (Catapano et al., 2016; Nordestgaard et al., 2016).

2.3. Biochemical characterisation of lipids and lipoproteins

The biochemical tests for TC, direct LDL-C, HDL-C, TG, apoA1, and apoB were performed by an enzymatic colorimetric and immunoturbidimetric method. Serum levels of sdLDL-C were measured by an enzymatic colorimetric method (sLDL-EX "Seiken"), and lipoprotein(a) [Lp(a)] by an immunoturbidimetric method, as previously described (Bourbon et al., *Submitted*).

2.4. Corrections factors regarding lipid-lowering therapy

Untreated lipid values (TC, LDL-C and apoB) for individuals under statins medication were estimated using correction factors: measured TC and LDL-C (Baigent et al., 2005; Peloso et al., 2014; Khera et al., 2016), as well as apoB (Sniderman, 2008), was divided by 0.8 (20% TC reduction), 0.7 (30% LDL-C reduction), and 0.763 (23.7% apoB reduction, corresponding to 79% of the LDL-C reduction), respectively. Untreated TG, HDL-C and apoA1 values were not estimated, since the effects of lipid-lowering therapy with statins apparently are not significant in these biomarkers (Schulzeck et al., 1988; Scandinavian Simvastatin Survival Study Group, 1994; Cannon, 2005; Kastelein et al., 2008).

2.5. General statistical analysis

Statistical analyses were performed using R (version 3.1.2) software (R: The R Project for Statistical Computing). For comparison analysis of lipids, lipoproteins and LDL-C GRS values between independent groups, the non-parametric Two-sample Wilcoxon or Kruskal-Wallis tests were applied for two or more independent samples, respectively. When there were assumptions of normality (Shapiro-Wilk or Kolmogorov-Smirnov tests) and homogeneity of variance (Bartlett test), the parametric ANOVA or Student t tests were applied for two or more independent samples. For comparison of proportions, the 95% confidence intervals (CI) were used. Whenever the two CI non-overlap, it was considered that there was evidence to conclude that the proportions are statistically different. In the remaining cases (overlap of the two proportions confidence intervals), the two proportions were compared using chi-square or Fisher's tests. The multiple of median (MoM) was calculated for the LDL-C, TG and apoB measured values to analyse how far those values deviate from the median of a reference population. The 50^{th} gender and age-specific percentile values for LDL-C, TG and apoB

previously estimated for the Portuguese population (Bourbon et al., Submitted) were used as reference values for MoM estimation.

3. RESULTS

3.1. Demographic and clinical data

Demographic and clinical data on cardiovascular disease of all index cases are shown in Table 1, including the lipid profile at study inclusion time, and the complete fasting lipid profile performed at our Institute for all individuals referred to the FH Study. Mean age (years) at inclusion was 9.94 (SD 3.69) for children and 45.67 (SD 13.32) for adults. The majority (>90%) of the patients are of Portuguese nationality distributed within all Portuguese regions.

3.1.1. FH positive versus FH negative

A total of 731 index cases was analysed as described in methods for *LDLR*, *APOB* and *PCSK9* genes. In 129 children and 154 adults a pathogenic or likely pathogenic variant was found; these will be referred as FH mutation positive (FH/M+). In 159 children and 239 adults no variants of relevance were found; these will be referred as FH mutation negative (FH/M-). Additionally, 18 children and 26 adults were found to have a VUS following American College of Medical Genetics and Genomics (ACMG) classification (Chora et al., 2017a).

Demographic, clinical and biochemical profile of FH/M+ were compared to FH/M-, for children and adults separately, and is presented in Tables 2-3, respectively. Patients with VUS were not included in this analysis, as well as homozygous patients. Although all FH/M- patients have a clinical phenotype of FH, they usually presented lower levels of TC, LDL-C, non-HDL-C, apoB, and apoB/apoA1 ratio, and higher levels of HDL-C and TG, than FH/M+. These differences are more evident in the paediatric cohort was considered. Also, the percentage of adult FH/M- patients with hypertriglyceridaemia (TG ≥200 mg/dL) was higher than for the FH/M+ patients (20.00% CI=[14.89%-25.11%] (n=47/235) *versus* 8.33% CI=[3.82%-12.85%] (n=12/44), P=0.002). The opposite was observed for high apoB (≥120 mg/dL) values, where it was higher in the FH/M+ patients (87.85% CI=[82.45%-93.27%] (n=123/140) *versus* 64.94% CI=[58.78%-71.09%] (n=150/231), P<0.001). No significant differences were observed for Lp(a) values.

The lipid values of FH/M+ index and FH/M+ relatives presented significant differences: TC, LDL-C, apoB, non-HDL-C and apoB/apoA1 ratio were statistically higher

in index cases, except in the paediatric cohort where the apoB values and apoB/apoA1 ratio did not differ (Supplementary Table 2).

Table 1 – Baseline characteristics of all index cases included in the Portuguese Familial Hypercholesterolaemia Study.

Clinical and demographic profile	Children (n=311)	Adults (n=420)
Age, years (SD)	9.94 (3.69)	45.67 (13.32)
Male gender, n (%)	133 (42.77)	191 (45.48)
BMI, kg/m^2 (SD)	19.67 (4.05)	26.06 (4.47)
Smoking, n (%)	1 (0.32)	82 (19.52)
Alcohol consumption, n (%)	0	135 (32.14)
Hypertension, n (%)	3 (0.97)	108 (25.71)
Diabetes, n (%)	1 (0.32)	15 (3.57)
Personal history of CVD, n (%)	0	93 (22.14)
Personal history of pCVD, n (%)	0	71 (16.90)
Family history of pCVD, n (%)	55 (17.69)	137 (32.62)
Pharmacological treatment (e.g. statins), n (%)	62 (19.94)	317 (75.48)
On diet, n (%)	145 (46.62)	48 (11.43)
Physically active, n (%)	223 (71.70)	145 (34.52)
Tendon xanthoma, n (%)	1 (0.32)	10 (2.38)
Corneal arcus, n (%)	0	15 (3.57)
Other xanthomas, n (%)	3 (0.97)	32 (7.62)
Lipid profile 1[a]		
Total cholesterol, mg/dL (IQR)	265.93 (242-290)	321.25 (296-362)
LDL-C, mg/dL (IQR)	189.00 (170-223)	243.00 (210-287)
HDL-C, mg/dL (IQR)	53.00 (45-62)	52.00 (44-63)
Triglycerides, mg/dL (IQR)	76.00 (57-109)	133.00 (96-185)
Lipid profile 2[b]		
Total cholesterol, mg/dL (IQR)	247.44 (227-284)	298.75 (259-348)
LDL-C, mg/dL (IQR)	178.00 (152-219)	222.00 (177-279)
HDL-C, mg/dL (IQR)	53.00 (45-63)	53.00 (44-64)
Triglycerides, mg/dL (IQR)	75.00 (56-108)	123.00 (91-164)
ApoB, mg/dL (IQR)	115.00 (94-140)	152.63 (119-191)
ApoA1, mg/dL (IQR)	143.00 (125-162)	152.00 (131-172)
Non-HDL-C, mg/dL (IQR)	189.69 (168-233)	244.00 (200-295)
ApoB/ApoA1 ratio, mg/dL (IQR)	0.84 (0.62-1.05)	0.98 (0.72-1.35)
Lp(a), mg/dL (IQR)	29.00 (10-70)	33.00 (9-75)

BMI, body mass index; CVD, cardiovascular disease; pCVD, premature cardiovascular disease; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1; Lp(a), lipoprotein(a); SD, standard deviation; IQR, interquartile range.

Age and BMI are expressed as mean (SD), and biochemical values are expressed as median (IQR).

[a]Lipid profile considered for inclusion in the Portuguese Familial Hypercholesterolaemia (FH) Study. Values used for the analysis were preferable values without medication at the time of inclusion in the Portuguese FH Study. When untreated values were not available, TC, LDL-C and apoB values under medication were corrected as described in the Material and Methods section of our study.

[b]Lipid profile determined in house. Correction factors were used to estimate untreated values for TC, LDL-C, and apoB, as described in the Material and Methods section of our study.

Table 2 – Clinical, demographic and biochemical profile of Familial Hypercholesterolaemia patients in the Portuguese Familial Hypercholesterolaemia Study: children index cases.

	Paediatric cohort (n=285)[a]		
Clinical and demographic profile	**FH/M-**	**FH/M+**	P value[c]
n (%)	159 (51.13)	126 (40.51)	ND
Age, years (SD)	9.77 (3.48)	10.03 (3.86)	0.575
Male gender, n (%)	63 (39.62)	63 (50.00)	0.103
BMI, kg/m^2 (SD)	**20.27 (4.29)**	**18.90 (3.51)**	**0.012**
Smoking, n (%)	0	1 (0.79)	ND
Alcohol consumption, n (%)	0	0	ND
Hypertension, n (%)	3 (1.89)	0	ND
Diabetes, n (%)	1 (0.63)	0	ND
Personal history of CVD, n (%)	0	0	ND
Personal history of pCVD, n (%)	0	0	ND
Family history of pCVD, n (%)	26 (16.35)	24 (19.05)	0.662
Pharmacological treatment (e.g. statins), n (%)	**18 (11.32)**	**36 (28.57)**	**<0.001**
On diet, n (%)	**86 (54.09)**	**50 (39.68)**	**0.021**
Physically active, n (%)	119 (74.84)	86 (68.25)	0.323
Tendon xanthoma, n (%)	0	0	ND
Corneal arcus, n (%)	0	0	ND
Other xanthomas, n (%)	0	1 (0.79)	ND
Lipid profile[b]			
Total cholesterol, mg/dL (IQR)	**239.21 (211-259)**	**273.38 (245-302)**	**<<0.001**
LDL-C, mg/dL (IQR)	**156.88 (135-178)**	**203.64 (5=179-238)**	**<<0.001**
HDL-C, mg/dL (IQR)	**57.19 (49-68)**	**50.98 (42-58)**	**<0.001**
Triglycerides, mg/dL (IQR)	**84.50 (65-119)**	**65.00 (54 -88)**	**<0.001**
ApoB, mg/dL IQR)	**99.50 (85-122)**	**129.50 (113-151)**	**<<0.001**
ApoA1, mg/dL (IQR)	**150.00 (136-174)**	**135.00 (115-147)**	**<<0.001**
Non-HDL-C, mg/dL (IQR)	**172.00 (153-194)**	**216.06 (192-253)**	**<<0.001**
ApoB/ApoA1 ratio, mg/dL (IQR)	**0.66 (00.52-0.82)**	**0.99 (0.85-1.18)**	**<<0.001**
Lp(a), mg/dL (IQR)	32.00 (10-80)	24.64 (10-44)	0.081

FH, Familial Hypercholesterolaemia; FH/M-, FH mutation negative; FH/M+, FH mutation positive; BMI, body mass index; CVD, cardiovascular disease; pCVD, premature cardiovascular disease; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1; Lp(a), lipoprotein(a); SD, standard deviation; IQR, interquartile range; ND, Not determined.

Age and BMI are expressed as mean (SD), while biochemical lipid values are expressed as median (IQR).

[a]Only heterozygous patients with pathogenic or likely-pathogenic variants were considered as FH/M+. Homozygous FH and patients with variants of uncertain significance (VUS) were excluded.

[b]Lipid profile determined in house. Correction factors were used to estimate untreated values for TC, LDL-C, and apoB, as described in the Material and Methods section of our study.

[c]Statistical significance (P value <0.05).

Table 3 – Clinical, demographic and biochemical profile of Familial Hypercholesterolaemia patients in the Portuguese Familial Hypercholesterolaemia Study: adult index cases.

	Adult cohort (n=386)[a]		
Clinical and demographic profile	**FH/M-**	**FH/M+**	P value[c]
n (%)	239 (56.90)	147 (35.00)	ND
Age, years (SD)	47.20 (11.91)	43.73 (14.91)	**0.008**
Male gender, n (%)	118 (49.37)	57 (38.78)	0.054
BMI, kg/m^2 (SD)	26.14 (4.21)	26.10 (4.86)	0.434
Smoking, n (%)	**56 (23.43)**	**21 (14.29)**	**0.025**
Alcohol consumption, n (%)	86 (35.98)	40 (27.21)	0.125
Hypertension, n (%)	61 (25.52)	41 (27.89)	0.572
Diabetes, n (%)	9 (3.77)	5 (3.40)	ND
Personal history of CVD, n (%)	51 (21.34)	31 (21.09)	0.999
Personal history of pCVD, n (%)	41 (17.15)	20 (13.61)	0.433
Family history of pCVD, n (%)	80 (33.47)	43 (29.25)	0.452
Pharmacological treatment (e.g. statins), n (%)	183 (76.57)	109 (74.15)	0.678
On diet, n (%)	30 (12.55)	14 (9.52)	0.457
Physically active, n (%)	81 (33.89)	52 (35.37)	0.696
Tendon xanthoma, n (%)	0	10 (6.80)	ND
Corneal arcus, n (%)	1 (0.42)	13 (8.84)	ND
Other xanthomas, n (%)	13 (5.44)	16 (10.88)	ND
Lipid profile[b]			
Total cholesterol, mg/dL (IQR)	**285.00 (242-321)**	**331.25 (284-395)**	**<<0.001**
LDL-C, mg/dL (IQR)	**201.71 (158-246)**	**263.57 (207-318)**	**<<0.001**
HDL-C, mg/dL (IQR)	55.00 (44-64)	51.00 (44-62)	0.517
Triglycerides, mg/dL (IQR)	**129.00 (100-178)**	**112.39 (83-148)**	**<0.001**
ApoB, mg/dL (IQR)	**136.84 (136-179)**	**177.63 (141-211)**	**<<0.001**
ApoA1, mg/dL (IQR)	**155.65 (136-179)**	**146.50 (127-167)**	**0.005**
Non-HDL-C, mg/dL (IQR)	**222.13 (182-270)**	**274.88 (224-328)**	**<<0.001**
ApoB/ApoA1 ratio, mg/dL (IQR)	**0.88(0.68-1.17)**	**1.16 (0.92-1.56)**	**<<0.001**
Lp(a), mg/dL (IQR)	30.00 (9-72)	35.15 (9-92)	0.339

FH, Familial Hypercholesterolaemia; FH/M-, FH mutation negative; FH/M+, FH FH/M+; BMI, body mass index; CVD, cardiovascular disease; pCVD, premature cardiovascular disease; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1; Lp(a), lipoprotein(a); SD, standard deviation; IQR, interquartile range; ND, Not determined.

Age and BMI are expressed as mean (SD), while biochemical lipid values are expressed as median (IQR).

[a]Only heterozygous patients with pathogenic or likely-pathogenic variants were considered as FH/M+. Homozygous FH and patients with variants of uncertain significance (VUS) were excluded.

[b]Lipid profile determined in house. Correction factors were used to estimate untreated values for TC, LDL-C, and apoB, as described in the Material and Methods section of our study.

[c]Statistical significance (P value <0.05).

3.2. Monogenic dyslipidaemia

3.2.1. Familial Hypercholesterolaemia

In 38.58% (n=283) of the 731 index patients, at least one pathogenic or likely pathogenic variant was identified in the *LDLR*, *APOB* and *PCSK9* genes. Additionally, 1 index patient was found with a stop mutation in the *APOE* gene. In the paediatric cohort (n=311), a total of 127 (40.84%) had heterozygous (HeFH) and 2 (0.64%) had homozygous FH (HoFH) (Figure 1). In the adult cohort (n=420), 35.00% (n=147) had HeFH and 1.67% (n=7) had HoFH (Figure 1). Also, VUS in the *LDLR* and *APOB* genes were found in 5.79% (n=18) of children and 6.19% (n=26) of adults, in a total of 35 and 9 patients with a VUS in the *LDLR* and *APOB* gene, respectively. The cascade-screening programme led to the additional identification of 121 HeFH children, and 309 HeFH and 1 HoFH adults. Additionally, 6 children and 32 adults presented with a VUS. Since our last report in 2015, 8 *LDLR* and 1 *APOE* novel variants have been identified (3 never described and 6 described in other countries, but novel for Portugal) and have already been submitted to ClinVAR (Supplementary Table 3). From these, only 3 are considered pathogenic or likely pathogenic: c.2214del/p.(Gln739Serfs*26), c.941-2A>C, and c.1897C>T/p.(Arg633Cys) (Supplementary Table 3).

3.2.2. Other monogenic causes

As described in methods, selected FH/M- patients were analysed for other possible causes of monogenic dyslipidaemia. In 6 index patients, 5 children and 1 adult, mutations in the *APOE*, *LIPA*, *ABCG8* and albumin (*ALB*) genes were found. Three children had lysosomal acid lipase deficiency (LALD) (due to c.894G>A variant in *LIPA*) (Chora et al., 2017b), and 1 had sitosterolemia (due to c.1974C>G variants in *ABCG8*), 1 had congenital analbuminemia (due to c.1289+1G>A variant in *ALB*). The molecular identification of the variant in *ALB* gene was not performed by our group (Caridi et al., 2012). In the adult cohort, 1 individual had an autosomal dominant hypercholesterolaemia (due to c.487C>T variant in *APOE*). Additionally, 1 relative was found to have LALD.

Overall, monogenic dyslipidaemia is responsible for 39.53% (n=289/731) of all index cases with an FH phenotype. Within all these cases with a monogenic cause of hypercholesterolaemia, 89.55% of children and 93.51% of adults had a mutation causing disease in *LDLR*, 4.48% of children and 5.20% of adults in *APOB*, 0.75% of children and 1.30% of adults in *PCSK9*, 2.24% of children in *LIPA*, 0.65% of adults in *APOE*, 0.75% of

children in *ABCG8*. All together, other monogenic causes represent 2.08% of all index cases with a significant proportion in children (3.73%).

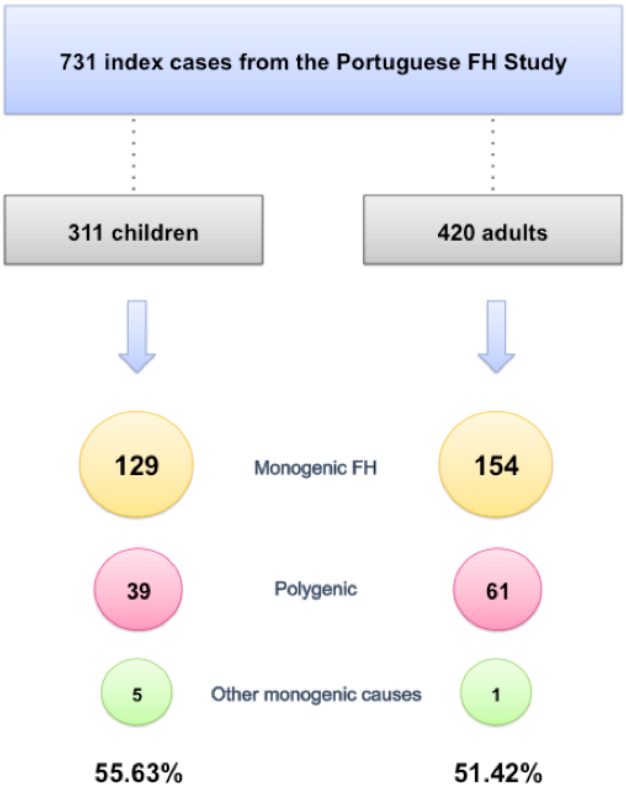

Figure 1 – Number of index cases, children and adults, presenting with the FH phenotype from the Portuguese Familial Hypercholesterolaemia (FH) Study and number of individuals with a genetic identified cause for their dyslipidaemia. Monogenic FH refers to patients with mutations in either *LDLR, APOB* or *PCSK9*, polygenic to patients with a LDL genetic risk score (GRS) above 0.76 (6 SNPs GRS), and other causes are: in children, 3 with lysosomal acid lipase deficiency (mutation in the *LIPA* gene), 1 with sitosterolaemia (mutation in the *ABCG8* gene) and 1 with analbuminemia (mutation in *ALB* gene); in adults, 1 with an autosomal dominant hypercholesterolaemia (mutation in the *APOE* gene).

3.3. Polygenic hypercholesterolaemia

3.3.1. LDL-C genetic risk score

The mean value of the LDL-C GRS calculated in e_COR controls was 0.62 (SD 0.22) with a mean LDL-C of 135.75 mg/dL (SD 46.34) [3.51 mmol/L (SD 1.20)]. Distribution of the LDL-C concentration values by percentiles showed that individuals above the P75th (LDL-C GRS 0.76) had higher LDL-C (P<<0.001) than individuals below the 25th percentile (P25th) (LDL-C GRS 0.51) (Supplementary Figure 1). The LDL-C, apoB, non-HDL-C, and sdLDL-C were demonstrated to be the most correlated lipid biomarkers with the weighted LDLC GRS, showing that mean concentration values increase with the increase of the weighted LDL-C GRS (Supplementary Tables 4-5). When comparing the score values between Portuguese e_COR and UK Whitehall II (WHII) controls (Talmud et al., 2013), no differences were seen (0.62 (SD 0.22) CI=[0.61-0.63] *versus* 0.63 (SD 0.22) CI=[0.62-0.64], respectively).

Of all 731 clinical FH index cases, the LDL-C GRS were calculated for 455 individuals from whom DNA was available, 186 children and 269 adults. Compared with e_COR controls, FH/M- and FH/M+ patients had higher LDL-C GRS (P<<0.001). However, no statistically significance differences were seen comparing FH/M- and FH/M+ patients. In the children cohort, the mean value was 0.73 (SD 0.17) for FH/M- and 0.71 (SD 0.19) for FH/M+, and in the adult cohort was 0.72 (SD 0.19) for FH/M- and 0.69 (SD 0.20) for FH/M+.

3.3.2. Polygenic hypercholesterolaemia versus monogenic dyslipidaemia

About 40.39% CI=[32.57%-48.22%] (n=61) of adult FH/M- patients are above the P75th (>0.76) and 10.60% CI=[5.69%-15.51%] (n=16) are below the P25th (≤0.51) for the LDL-C GRS, respectively. For FH/M+ adult patients, 34.07% [CI=24.33%-43.80%] (n=31) are above the P75th and 19.78% [CI=11.60%-27.96%] (n=18) are below the P25th. In the paediatric FH/M- cohort, about 42.39% CI=[32.29%-52.49%] (n=39) of patients are above P75th and 13.04% CI=[6.16%-19.93%] (n=12) of patients was below the P25th (P<0.001), while 39.47% CI=[28.48%-50.46%] (n=31) of FH/M+ patients are above P75th and 15.79% CI=[7.59%-23.99%] (n=12) of patients are below P25th. Because individuals with LDL-C GRS above the P75th were considered to have polygenic hypercholesterolaemia, this means that from all 398 FH/M- patients (159 children and 239 adults), a polygenic cause can be attributed to 39 children and 61 adults, giving a polygenic dyslipidaemia diagnosis rate of 12.54% (n=39/311) in children and 14.52% (n=61/420) in adults. Of the remaining

298 FH/M- patients, 9.40% (n=28) presented a low score (below P25th) and could have other known or unknown cause of monogenic dyslipidaemia (12 children and 16 adults).

We then evaluated the LDL-C, TG and apoB levels in FH/M- and FH/M+ patients according to the bottom (P25th) and top (P75th) percentiles for the LDL-C GRS. Although concentration values for these lipid biomarkers were higher for both FH/M- and FH/M+ individuals above the P75th comparing to P25th (except for TG in FH/M+ individuals, which was modestly higher in individuals below the P25th), no statistically significant differences were found in adults; in children, a statistically significant difference was only seen for apoB values in FH/M- patients (Table 4). Although it was observed that LDL-C and apoB values are higher in FH/M+ than in FH/M-, both for adults and children, for both P25th and P75th no statistically significant differences were seen. The inverse was observed for TG values, with FH/M- patients presenting higher values than FH/M+, but again without statistically significant differences. Nonetheless, when analysing how far the LDL-C, TG and apoB values deviates from the median of a reference population, differences were found between FH/M+ and FH/M- patients below P25th and above P75th for LDL-C and apoB, but not for TG: P25th (LDL-C (P=0.048); apoB (P=0.017; TG (P=0.171)) and P75th (LDL-C (P<0.001); apoB (P<0.001); TG (P=0.313)).

Table 4 – Evaluation of LDL-C, apoB and triglycerides concentration in Familial Hypercholesterolaemia mutation negative and mutation positive index patients below 25th and above 75th percentiles for the LDL-C genetic risk score.

		Children			Adults		
Lipid Biomarker	**FH Study**	**P25th**	**P75th**	P value[a]	**P25th**	**P75th**	P value[a]
LDL-C	FH/M-	140.50 (39.50)	159.00 (40.87)	0.169	175.86 (108.72)	194.34 (94.48)	0.514
	FH/M+	184.74 (62.50)	205.50 (65.50)	0.802	230.00 (82.86)	240.13 (122.30)	0.604
ApoB	FH/M-	**79.00 (29.25)**	**95.50 (30.68)**	**0.018**	113.03 (56.25)	134.11 (56.79)	0.192
	FH/M+	122.37 (39.38)	127.44 (39.83)	0.513	143.42 (50.11)	174.34 (69.57)	0.278
Triglycerides	FH/M-	81.00 (45.00)	90.00 (65.00)	0.433	126.00 (76.00)	131.00 (91.43)	0.959
	FH/M+	85.00 (70.43)	69.91 (48.84)	0.713	113.50 (92.50)	103.54 (70.89)	0.894

FH, Familial Hypercholesterolaemia; FH/M-, FH mutation negative; FH/M+, FH mutation positive; P25th, percentile 25th; P95th, percentile 95th; GRS, genetic risk score; LDL-C, low-density lipoprotein cholesterol; ApoB, apoliporpotein B LDL-C, low-density lipoprotein cholesterol; ApoB, apoliporpotein B.

Biochemical lipid values are expressed as mean (SD).

[a]Comparison between FH/M- and FH/M+ patients below the P25th and above the P75th for the LDL-C GRS; Statistical significance (P value <0.05).

We also examined the body mass index (BMI) in top percentiles for the LDL-C GRS and there is a statistically significant difference between the proportions of adult FH/M- patients with overweight/obesity above the P75th and below the P25th (70.00% CI=[57.30%-82.70%] (n=35/50) *versus* 33.33% CI=[9.48%-57.19%] (n=5/15), respectively). However, when comparing above the children 85th percentile for BMI, no differences were found for FH/M- patients above the P75th (55.56% CI=[39.32%-71.79%] (n=20/36)), and below the P25th (45.45% CI=[16.03%-74.48%] (n=5/11)), P=0.555). Furthermore, we analysed the percentage of all monogenic and polygenic causes in two age groups and found that individuals aged below 30 years (n=364) had statistically significant higher percentage of an identifiable monogenic cause for hypercholesterolaemia than individuals aged above 30 years (46.15% CI=[41.03%-51.28%] (n=168) *versus* 32.96% CI=[28.09%-37.83%] (n=118), respectively). When looking for polygenic causes, the percentage was slightly lower for patients below 30 years, but not significantly (11.81% CI=[8.50%-15.13%] (n=43) *versus* 14.53% [CI=10.88%-18.18%] (n=52), respectively, P=0.280). Specifically, mean LDL-C GRS were the same for these age groups, 0.71 (SD 0.19) for young individuals and 0.71 (SD 0.18) for aged above 30 years.

3.3.3. Simon Broome versus Dutch Lipid Network clinical FH criteria – monogenic and polygenic hypercholesterolaemia

All adult index cases included in this study fulfilled SB criteria prior inclusion, 410 possible FH and 10 definite FH. The DLCNS was applied to 382 of the 420 adults from the Portuguese FH Study. For the remaining 38 it was not possible to estimate the DLCNS value, due to previous missing LDL-C values. Main results for lipid profile according to the DLCNS values are shown in Supplementary Table 5. Eight individuals were classified as unlikely (DLCNS <3), 131 as possible FH (DLCNS 3-5), 169 as probable FH (DLCNS 6-8), and 82 as definite FH (DLCNS >8). The identification rate of the cause of dyslipidaemia increases according to the DLCNS, giving a diagnostic detection rate of dyslipidaemia of 35.88%, (n=47/131) within possible FH, 55.03% within probable FH (n=93/169), and 68.29% (n=56/82) within definite FH. Comparison analysis of the monogenic and polygenic dyslipidaemia genetic diagnosis rate between DLCN and SB clinical criteria showed no statistically significant differences for both monogenic and polygenic total diagnosis rate (Table 5). However, statistically significant differences were found when comparing clinical diagnosis FH groups. In summary, monogenic diagnosis rate is higher for both possible FH (P=0.024) and definite FH (P=0.027) groups of SB criteria, while in the polygenic hypercholesterolaemia no differences were observed for the

possible/probable FH group. For the individuals classified as definite FH, only some of those from DLCN criteria were found as having LDL-C GRS above the P75th; none definite FH classified according to the SB criteria had polygenic hypercholesterolaemia, but no statistical inference was performed due to very small sample size.

Table 5 – Genetic diagnosis rate of monogenic dyslipidaemia and polygenic hypercholesterolaemia according to the Dutch Lipid Clinic Network and Simon Broome criteria for clinical diagnosis of Familial Hypercholesterolaemia.

	Monogenic dyslipidaemia						
	DLCN[a]			**Simon Broome[b]**			
Clinical diagnosis	N	n (%)	95% CI	N	n (%)	95% CI	**P value[d]**
Possible/Probable FH	300	**89 (29.67)**	24.5-34.84	410	**155 (37.80)**	33.11-42.50	**0.024**
Definite FH	82	**52 (63.41)**	52.99-73.84	10	**10 (100.00)**	72.25-100.00	**0.027**
Total	382	141 (36.91)	32.07-41.75	420	165 (39.29)	34.61-43.96	0.490
	Polygenic hypercholesterolaemia						
	DLCN[a]			**Simon Broome[b]**			
Clinical diagnosis	N[c]	n (%)	95% CI	N[c]	n (%)	95% CI	**P value[d]**
Possible/Probable FH	194	51 (26.29)	20.09-32.48	266	61 (22.93)	17.88-27.98	0.406
Definite FH	48	4 (8.33)	2.27-20.87	4	0	0.00-48.99	ND
Total	276	55 (19.93)	15.21-24.64	270	61 (22.59)	17.60-27.58	0.447

FH, Familial Hypercholesterolaemia; LDL-C, low-density lipoprotein cholesterol; DLCN, Dutch Lipid Clinic Network; CI, confidence interval; ND, not determined.

Genetic diagnosis rate of monogenic and polygenic causes for hypercholesterolaemia are presented as n (%).

[a]Possible and probable FH clinical diagnosis were grouped for comparison analysis with Simon Broome criteria.

[b]All patients included in the Portuguese FH Study full field Simon Broome clinical criteria.

[c]Sample size for whom LDL-C genetic risk score (GRS) was calculated.

[d]Comparison of genetic diagnosis rate between DLCN and Simon Broome clinical criteria. Statistical significance (P value <0.05).

4. DISCUSSION

Correctly identifying the cause of dyslipidaemia

From all individuals included in the Portuguese FH Study, we have identified 714 patients with disease-causing variants and 82 patients with VUS, in one of the three genes associated with FH. From 2014 to 2016, a total of 10 novel variants were identified in our cohort, 3 never described before in association with FH. Previously identified VUS and these novel variants are being functionally characterised for correct pathogenicity classification since the lack of *in vitro* analysis can lead to misdiagnosis (Benito-Vicente et al., 2015). Only after functional characterisation is performed, is the

clinician informed that the variant found is the cause of disease. Our group has already successfully characterised 52 variants, improving this way the genetic diagnosis of FH.

Based on the estimated frequency of 1:500 of HeFH in Europe, up to date our study identified 3.6% of the estimated HeFH patients to exist in the Portuguese population. If VUS variants are proved to be pathogenic, an increase to 4.0% will be observed. These numbers although small, place Portugal in the top 10 of countries with more percentage of patients identified (Nordestgaard et al., 2013).

In some individuals with FH phenotype, no monogenic or a polygenic cause could be identified. Wang and colleagues (Wang et al., 2016) raise the hypothesis that, in these cases, the FH phenotype could be due to variants in other genes yet to be described or other genes of lipid metabolism, interactions between known genes, variants inaccessible by the currently sequencing techniques, epigenetics or even environmental factors *per se*. Taking this into consideration, our group started to analyse other monogenic or polygenic causes in patients with a clinical diagnosis of FH, but without a known FH-mutation. Indeed, in this work other genes associated to monogenic dyslipidaemia were investigated, namely *LIPA* (Chora et al., 2017a), *APOE* (Wintjesns et al., 2016), and *ABCG8* (Rios et al., 2010) genes, leading to a successful identification of the cause of dyslipidaemia in 6 cases. Overall, from a total of 731 index cases with clinical FH following SB criteria, in 39.53% (n=289) we have found a monogenic cause of disease: 274 having HeFH, 9 having HoFH, and 6 having other lipid related disorders. This shows that in patients with a clinical diagnosis of FH without FH-causing mutation, other monogenic cause could be involved. Other cases have been identified before (Stitziel et al., 2013), but their relation to the FH phenotype had not been established before. Interestingly, in patients with an identified mutation, 91.70% have a mutation in *LDLR*, 4.84% in *APOB*, 1.04% in *PCSK9* and 2.08% have other causes (*LIPA* 1.04%, *APOE* 0.35%, *ABCG8* 0.35% and *ALB* 0.35%). Other causes are more prevalent in children representing 3.73% of all cases. It is worth noting that other causes are more prevalent than *PCSK9* mutations, reinforcing the need to study these FH phenocopy genes for a more accurate patient diagnosis and management. There are considerable differences in the treatment of these several phenocopies which, if identified, can lead to a better patient prognosis.

Another interestingly result was the similar apoB and apoB/apoA1 ratio values for both index and relatives FH/M+ patients in children. In fact, the same results were found previously, reinforcing the idea that these biomarkers might be useful in a routine practice to improve at risk patient identification for a better stratification, management and prognosis in a paediatric population.

Apart from monogenic causes, we also investigated polygenic hypercholesterolaemia in our cohort. We have validated the LDL-C GRS in the Portuguese population, using the 6 LDL-C-raising SNPs previously defined by Talmud et al. (Talmud et al., 2013) and Futema et al. (Futema et al., 2015). We have found no difference in the score values between UK WHII and Portuguese e_COR controls. Our results were consistent with the fact that FH negative patients have higher LDL-C GRS than individuals from the general population, showing that their LDL-C plasma levels could be due to the influence of a combination of several LDL-C variants, each with modest effect. When the percentage of individuals below the lowest (25th) and above the highest (75th) percentiles for the LDL-C GRS was evaluated, the majority of FH negative patients were in the fourth quarter (>P75th), being considered to have polygenic hypercholesterolaemia (13.68% (n=100)). Similar proportion of patients above the highest percentile was also found in the FH/M+ group. They also had higher LDL-C GRS than e_COR controls, and as previously suggested (Talmud et al., 2013; Futema et al., 2015; Sharifi et al., 2017), FH phenotype in FH/M+ patients could being modulated by the modest effect of these LDL-C raising variants, at least at some level. Interestingly, although LDL-C in FH patients above the P75th was higher than in the P25th, this difference did not seem to be statistically significant, which could be due insufficient statistical power due to small sample size. As have been suggested by previously studies (Futema et al., 2015; Wang et al., 2016), another explanation is that those FH/M- individuals below the P25th for the LDL-C GRS may have an unidentified monogenic dyslipidaemia with a large effect on LDL-C values. In the case of FH/M+ patients, where a single mutation has been already identified, their effect could overlap the effect of several LDL-C-raising variants with modest effect explaining why several FH/M+ patients also have high GRS. These results were observed in both the paediatric and adult cohort.

In resume, from all index cases referred to the Portuguese FH Study, in 53.21% (n=389) we have identified the cause of hypercholesterolaemia: 39.53% (n=289), with monogenic hypercholesterolaemia and 13.68% with polygenic hypoercholesterolaemia, If all VUS are indeed pathogenic (found in 44 patients (6.02%)), the total number of positives cases with a monogenic disorder will increase to 45.55% and the total identification rate to 59.23%. In this context, it is reasonable to say that the genetic diagnosis of FH could benefit from the inclusion of all these genes studied in this work and the LDL-C GRS SNPs in a next-generation target panel without a great increase in cost. Actually, a panel with the 3 proven FH-causing genes, *LDLR*, *APOB*, and *PCSK9*, and also 3 known phenocopies, *LIPA*, *APOE* and *LDLRAP1* genes, and LDL-C GRS (6 SNPs)

has been implemented in our group, being the inclusion of the sitosterolaemia genes planned for the near future.

An unknown dyslipidaemia in the FH mutation negative patients

In the 298 (40.77%) FH/M- patients with no identified cause for their hypercholesterolaemia, about 9.40% (n=28) presented a low LDL-C GRS (below P25th) and could have other known or unknown cause of monogenic dyslipidaemia. We still believe that, in a small fraction of these patients, a new gene causing FH is yet to be discovered, although other assumptions should be considered, such as interactions between known genes or epigenetics, but in the majority of the cases environmental factors *per se* could be the cause of the phenotype (Wang et al., 2016). As shown here, our FH/M+ patients had higher lipid values when compared with FH/M- patients, with exception of TG, so the majority of our FH/M- patients will probably not have a monogenic disorder of lipid metabolism. The polygenic/environmental hypercholesterolaemia is more easily modulated by life habits, and our results are in line with that, showing that TG values seem to be higher within FH/M- patients with higher LDL-C GRS. Although no statistically significant differences were seen, once again this could simply reflect the small sample size. In addition, there were more adults with BMI above 25 kg/m^2 in the highest percentile for LDL-C GRS. However, the same was not observed for children above the 85th percentile for BMI. For instance, it was previously demonstrated by Medeiros et al. (2014) (Medeiros et al., 2014) that in our paediatric cohort, even the use of an exclusion criteria of BMI above the 75th percentile does not add benefits to the differentiation between FH and polygenic and/or environmental dyslipidaemia, so this result was not surprisingly. It is known that a genetic diagnosis rate of FH is usually higher in young patients, so we wondered if for the polygenic hypercholesterolaemia it would be the opposite. In fact, the monogenic diagnosis rate was statistically significantly higher in patients aged below 30 years, when compared to those above 30 years old. Nevertheless, the difference in percentage of polygenic diagnosis in younger and older patients was modest, only 2.71% of difference with no statistical significance.

S*crutinizing the diagnostic rate using different clinical FH criteria*

The DLCN and SB are the current clinical criteria for FH mostly used in Europe. However, in contrast to the SB, DLCN criteria do not present specific cholesterol levels for children, hence it was not applied to our children cohort in this present study for criteria comparison. It was previously showed that both clinical criteria do not differ greatly (Alves et al., 2012), so we explored their diagnosis rate for monogenic dyslipidaemia and polygenic hypercholesterolaemia and also found no great differences. However, looking at

clinical diagnosis criteria, the positive genetic diagnosis rate of SB is greater than in DLCN As expected, the monogenic dyslipidaemia diagnosis rate increase and polygenic hypercholesterolaemia clinical diagnosis rate decrease according to "possible FH"/"probable FH" and "definite FH" clinical diagnosis.

Importance of distinguishing the different causes of dyslipidaemia

Monogenic dyslipidaemias present a severe phenotype and are associated with an elevated CVD risk *per se*, like FH, while mild to severe dyslipidaemias are mostly due to polygenic hypercholesterolaemia, as a result of various genetic alterations that may interact, being modulated by non-genetic factors as life style. The distinction between these two types of dyslipidaemia is important for patient cardiovascular risk assessment and therapeutic management. It has been shown that FH patients with a pathogenic variant have 16 times greater cardiovascular risk compared to another individual with the same LDL value (Khera et al., 2016), but their life expectancy can be increased if they are identified early and treated correctly. This shows the importance of correctly identifying the cause of dyslipidaemia and to address other cardiovascular risk factors in childhood, to reduce CVD later in adulthood. The Portuguese FH Study will continue to investigate the genetic complexity of FH, as well as other monogenic and polygenic causes of hypercholesterolaemia, in order to contribute to a more personalised counselling and treatment of these patients.

5. CONCLUSIONS

The FH phenotype comprehends different genotypes, especially in paediatric cohorts. The correct identification of the cause of the dyslipidaemia is important for patient management and implementation of the best therapeutic measures for the best patient prognosis. We recommended that the genetic test for the identification of the genetic cause of the hypercholesterolaemia in clinical FH patients should include all the genes described here for the most effective patient diagnosis. Investigation on other genes causing the FH phenotype should be encouraged. The LDL-C GRS was validated in the Portuguese population and revealed that almost half of the FH negative patients (41.15%) could have polygenic hypercholesterolaemia, while a small part have a low score (11.52%), which could mean that these patients have an unknown variant in a new gene and should be investigated by exome sequencing. Finally, the evaluation of monogenic and polygenic diagnosis rate between DLCN and SB criteria suggests that although they present different positive diagnosis rates within subgroups, the overall results indicate that they do not have significant different discriminative power.

6. STUDY LIMITATIONS

For some comparison analysis in the polygenic dyslipidaemia, the small sample size could imply bias. Although it is considered as a limitation of our study, our results are in line with the previously reported (Talmud et al., 2013; Futema et al., 2015). Another limitation of our study is that we have used the adult controls values for the paediatric cohort comparison. To confirm our results, comparison should be made against child controls. Also, the reduction in TC, LDL-C and apoB that we accounted for in those undergoing lipid-lowering therapy might imperfectly estimate the untreated values due to the heterogeneity in drug response, dosing and variability in baseline lipid values. However, the 30% reduction in LDL-C and 20% in TC was implemented in previous studies (Baigent et al., 2005; Sniderman, 2008; Khera et al., 2016).

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8. SUPPLEMENTARY DATA

8.1. Supplementary Tables

8.1.1. Supplementary Table 1

Supplementary Table 1 – The 6 LDL-C genetic risk score SNPs.

Gene	Chr	SNP	Haplotype[a]	Risk allele	Effect size[b]
CELSR2	1	rs629301	..	T	0.15
APOB	2	rs1367117	..	A	0.10
ABCG8	2	rs4299376	..	G	0.071
LDLR	19	rs6511720	..	G	0.18
APOE	19	rs429358	ε2ε2	..	-0.90
		rs7412	ε2ε3	..	-0.40
			ε2ε4	..	0.20
			ε3ε3	..	0
			ε3ε4	..	0.10
			ε4ε4	..	0.20

Chr, chromosome; SNP, single nucleotide polymorphism.

[a]The *APOE* SNPs (rs7412 and rs429358) determined the *APOE* haplotype, an important genetic determinant of LDL-C levels, by resulting in different isoforms of the apolipoprotein E (ApoE): ε2, ε3, and ε4.

[b]Effect sizes are the beta coefficients reported by the Global Lipid Genetic Consortium for each minor allele, taken from previous studies (Talmud et al., 2013; Futema et al., 2015). Effect sizes are presented as mmol/L.

8.1.2. Supplementary Table 2

Supplementary Table 2 – Comparison of the lipid profile between index cases and relatives Familial Hypercholesterolaemia mutation positive patients of the Portuguese Familial Hypercholesterolaemia Study: adult and paediatric cohorts.

Adults cohort	Index	Relatives	
Lipid profile[a]	**FH/M+**	**FH/M+**	P value[b]
Total cholesterol, mg/dL (IQR)	**331.25 (284-395)**	**299.00 (256-339)**	**<0.001**
LDL-C, mg/dL (IQR)	**263.57 (207-318)**	**225.71 (1867-267)**	**<0.001**
HDL-C, mg/dL (IQR)	51.00 (44-62)	51.00 (43-62)	0.354
Triglycerides, mg/dL (IQR)	112.39 (83-148)	103.27 (71.39-156)	0.601
ApoB, mg/dL (IQR)	**177.63 (141-211)**	**156.29 (129-183)**	**<<0.001**
ApoA1, mg/dL (IQR)	146.50 (127-167)	143.00 (125-164)	0.442
Non-HDL-C, mg/dL (IQR)	**274.88 (224-328)**	**248.25 (205-294)**	**0.021**
ApoB/ApoA1 ratio, mg/dL (IQR)	**1.16 (0.92-1.56)**	**1.06 (0.84-1.33)**	**0.005**
Lp(a), mg/dL (IQR)	35.15 (9-91)	30.00 (14-77)	0.314
Paediatric cohort	Index	Relatives	
Lipid profile[a]	**FH/M+**	**FH/M+**	P value[b]
Total cholesterol, mg/dL (IQR)	**273.38 (245-302)**	**257.48 (219-294)**	**0.004**
LDL-C, mg/dL (IQR)	**203.64 (179-238)**	**196.00 (154-231)**	**0.002**
HDL-C, mg/dL (IQR)	50.98 (42-58)	50.00 (42-56)	0.608
Triglycerides, mg/dL (IQR)	65.00 (54-88)	71.50 (59-102)	0.081
ApoB, mg/dL (IQR)	129.50 (113-151)	123.76 (102-150)	0.102
ApoA1, mg/dL (IQR)	135.00 (115-147)	132.00 (114-143)	0.219
Non-HDL-C, mg/dL (IQR)	**216.06 (192-253)**	**210.44 (165-243)**	**0.021**
ApoB/ApoA1 ratio, mg/dL (IQR)	0.99 (0.85-1.18)	0.94 (0.75-1.16)	0.957
Lp(a), mg/dL (IQR)	24.64 (10-44)	27.00 (12-53)	0.609

FH, Familial Hypercholesterolaemia; FH/M-, FH mutation negative; FH/M+, FH mutation positive; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1; Lp(a), lipoprotein(a); SD, standard deviation; IQR, interquartile range.

Bochemical lipid values are expressed as median (IQR).

[a]Lipid profile determined in house. Correction factors were used to estimate untreated values for TC, LDL-C, and apoB, as described in the Material and Methods section of our study.

[b]Comparison of FH/M+ patients (HeFH) between index cases and relatives; Statistical significance (P value <0.05).

8.1.3. Supplementary Table 3

Supplementary Table 3 – Novel variants identified in the Portuguese Familial Hypercholesterolaemia Study between 2014 and 2016.

Gene	DNA alteration	Protein alteration	dbSNP	Functional studies		Pathogenicity[a]	ClinVar ID	Described	
APOE	c.683G>A	p.(Trp228*)	rs121918396	Yes	Lohse et al., 1992	P	17862	Described	Ghiselli et al., 1981
APOE	c.487C>T	p.(Arg163Cys)	rs769455	No	..	LP/P	17851	Described	Rall et al., 1983
LDLR	c.*13A>G	..	rs72658871	No	..	VUS	265909	Novel	..
LDLR	c.1499T>C	p.(Val500Ala)	rs886039833	No	..	VUS	265903	Novel	..
LDLR	c.2214del	p.(Gln739Serfs*26)	..	No	..	P	..	Novel	..
LDLR	c.1434G>A	p.(Gly478Gly)/p.(=)	rs886039832	No	..	VUS	265902	Novel	..
LDLR	c.1186+56_1186+64del	p.=	..	Yes	Bourbon et al., *not published*	LB	..	Novel	..
LDLR	c.1897C>T	p.(Arg633Cys)	rs746118995	No	..	LP	226379	Described	Day et al., 1997
LDLR	c.313+5G>A	p.(Leu64_Pro105delinsSer)	rs879254467	Yes	Liguori et al., 2001	VUS	251136	Described	Liguori et al., 2001
LDLR	c.941-2A>C	..	rs112366278	No	..	P	251553	Described	Chmara et al., 2010

dbSNP, Single Nucleotide Polymorphism database; ID, identification number; VUS, variant of unknown significance; LP, likely pathogenic; P, pathogenic; LB, likely benign.

[a]American College of Medical Genetics and Genomics (ACMG) classification for *LDLR* variants according to Chora et al., 2017 (Chora et al., 2017a).

8.1.4. Supplementary Table 4

Supplementary Table 4 – Pearson correlation of lipid and lipoprotein biomarkers with the LDL-C genetic risk score in the e_COR population.

	Correlation of lipid biomarkers with the weighted LDL-C genetic risk score (GRS)							
	LDL-C	**TC**	**TG**	**HDL-C**	**ApoB**	**ApoA1**	**Non-HDL-C**	**sdLDL-C**
***r*[a]**	**0.21**	**0.19**	-0.034	-0.05	**0.22**	**-0.06**	**0.2**	**0.12**
95% CI	0.16-0.26	0.13-0.24	-0.09-0.02	-0.10-0.004	0.17-0.27	-0.12-0.01	0.14-0.25	0.07-0.18
P value[b]	**<<0.001**	**<<0.001**	0.221	0.070	**<<0.001**	0.020	**<<0.001**	**<<0.001**

LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1; Non-HDL-C, non-high-density lipoprotein cholesterol; sdLDL-C, small, dense low-density lipoprotein cholesterol.

[a]Pearson correlation coefficient.

[b]Statistical significance (P value <0.05).

8.1.5. Supplementary Table 5

Supplementary Table 5 – LDL-C, apoB, non-HDL-C and sdLDL-C lipid profile of the Portugal control e_COR sample by LDL-C genetic risk score percentiles.

LDL-C and apoB				**LDL-C**		**ApoB**	
		Score value		mean (SD)		mean (SD)	
Percentile	n	mmol/L	Range	mmol/L	mg/dL	mmol/L	mg/dL
25th	380	0.51	-0.39-0.51	3.20 (1.17)	123.70 (45.33)	0.0018 (0.006)	94.17 (31.15)
50th	245	0.65	0.51-0.65	3.55 (1.11)	137.24 (43.10)	0.0020 (0.006)	104.12 (30.69)
75th	392	0.76	0.65-0.76	3.59 (1.17)	139.00 (45.06)	0.0020 (0.006)	105.08 (32.21)
100th	301	1.10	0.76-1.10	3.76 (1.26)	145.51 (48.79)	0.0021 (0.007)	109.87 (33.72)
Total	1318	..	-0.39-1.10	3.51 (1.20)	135.75 (46.34)	0.0020 (0.006)	102.85 (32.49)
Non-HDL-C and sdLDL-C				**non-HDL-C**		**sdLDL-C**	
		Score value		mean (SD)		mean (SD)	
Percentile	n	mmol/L	Range	mmol/L	mg/dL	mmol/L	mg/dL
25th	380	0.51	-0.39-0.51	3.70 (1.27)	143.12 (49.17)	0.71 (0.34)	27.30 (13.15)
50th	245	0.65	0.51-0.65	4.07 (1.23)	157.41 (47.47)	0.77 (0.35)	29.78 (13.68)
75th	392	0.76	0.65-0.76	4.09 (1.27)	158.15 (48.96)	0.78 (0.37)	30.12 (14.28)
100th	301	1.10	0.76-1.10	4.28 (1.35)	165.56 (52.37)	0.81 (0.40)	31.22 (15.42)
Total	1318	..	-0.39-1.10	4.02 (1.30)	155.37(50.20)	0.76 (0.37)	29.50 (14.19)

LDL-C, low-density lipoprotein cholesterol; ApoB, apolipoprotein B; Non-HDL-C, non-high-density lipoprotein cholesterol; sdLDL-C, small, dense low-density lipoprotein cholesterol.

LDL-C genetic risk score (GRS) and biochemical lipid values are expressed as mean (SD) in mmol/L and/or mg/dL.

8.1.6. Supplementary Table 6

Supplementary Table 6 – Baseline characteristics of adult index cases according to the Dutch Lipid Clinic Network clinical diagnosis criteria of Familial Hypercholesterolaemia.

	Dutch Lipid Clinic Network Score (DLCNS)			
	Unlikely	**Possible FH**	**Probable FH**	**Definite FH**
Lipid profile[a]	<3	3-5	6-8	>8
Total cholesterol, mg/dL (IQR)	241.63 (90.63)	266.25 (64.38)	307.50 (86.88)	378.65 (163.61)
LDL-C, mg/dL (IQR)	169.93 (66.12)	190.40 (70.71)	235.52 (97.47)	297.03 (178.91)
HDL-C, mg/dL (IQR)	55.50 (18.71)	53.67 (19.82)	53.00 (19.33)	51.37 (16.85)
Triglycerides, mg/dL (IQR)	96.46 (51.00)	119.50 (73.68)	115.97 (75.28)	120.00 (60.79)
Non-HDL-C, mg/dL (IQR)	185.38 (69.62)	212.45 (68.63)	255.25 (93.75)	318.48 (163.78)
Lp(a), mg/dL (IQR)	36.00 (80.30)	33.00 (66.48)	30.00 (70.00)	48.50 (78.48)
ApoB, mg/dL (IQR)	107.89 (36.84)	126.32 (42.21)	161.84 (64.47)	186.84 (102.24)
ApoA1, mg/dL (IQR)	155.00 (36.00)	155.50 (37.00)	153.50 (40.25)	141.00 (36.00)
ApoB/ApoA1 ratio, mg/dL (IQR)	0.72 (0.50)	0.80 (0.37)	1.09 (0.48)	1.31 (0.99)

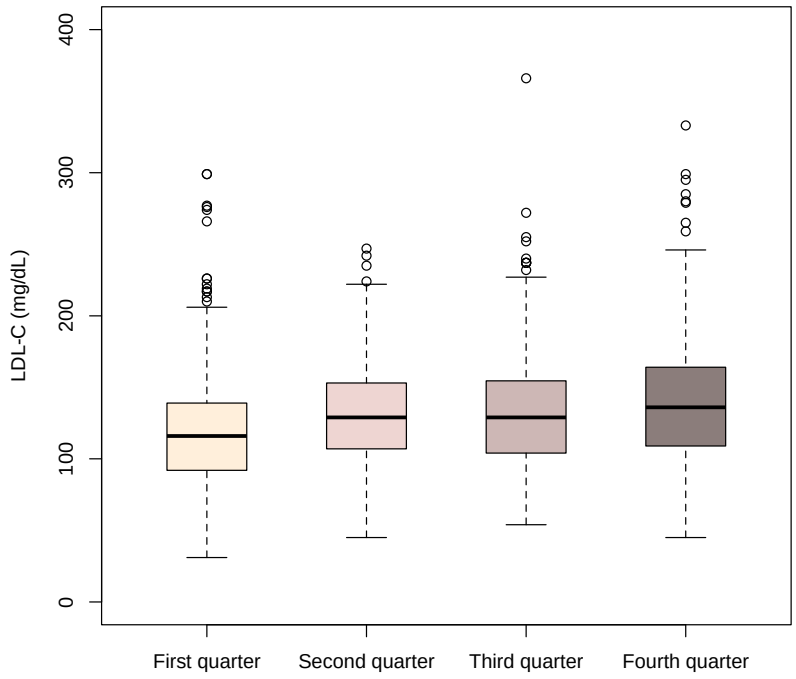
FH, Familial Hypercholesterolaemia; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA1, apolipoprotein A1; Lp(a), lipoprotein(a); SD, standard deviation; IQR, interquartile range.; Q1, first quartile; Q3, third quartile;

Biochemical lipid values are expressed median (IQR=Q3-Q1).

[a]Lipid profile determined in house. Correction factors were used to estimate untreated values for TC, LDL-C, and apoB, as described in the Material and Methods section of our study.

8.2. Supplementary Figures

8.2.1. Supplementary Figure 1



Supplementary Figure 1 – Boxplot of the LDL-C values distribution by e_COR population in the first (≤25th percentile), second (>25th≤50th percentile), third (>50th≤75th percentile), and fourth (>75th percentile) quarters of the LDL-C genetic risk score (GRS). Correction factors were used to estimate untreated values for LDL-C (a 30% of LDL-C reduction was considered for patients under treatment with statins). Individuals in the fourth quarter of the LDL-C GRS distribution had significantly higher LDL-C concentration than individuals in the first quarter ($P<<0.001$).

8.3. References

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Chapter 5

FINAL CONSIDERATIONS

1. CONCLUDING REMARKS AND PERSPECTIVES

The main goal of this thesis was to perform a complete characterisation of the dyslipidaemia in the Portuguese population, both biochemically and molecularly. In fact, dyslipidaemia is one of the major cardiovascular risk factors with both genetic and environmental determinants, where CVD-associated risks can be prevented by the modification of lifestyles and implementation of a correct treatment (Gielen and Landmesser, 2014). Since the identification of a public health problem is the first step to initiate preventive measures, knowing the dyslipidaemia profile of a population should contribute to the implementation of specific preventive interventions to change cardiovascular mortality trends.

One of the major contributions of this work was to determine, for the first time in Portugal, reference values for plasma TC, LDL-C, HDL-C, TG, apoA1, apoB, sdLDL-C, Lp(a), as well as for apoB/apoA1 and sdLDL-C/LDL-C ratios, and also non-HDL-C and remnant cholesterol based on the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles for the Portuguese population. These percentiles were determined by a rigorous methodology, using a bootstrap approach as a valid tool for percentiles estimation (Efron, 1979), and taking into account gender and age-specific stratum weights, based on Portuguese population distribution, that were used to overcome the limitation of the e_COR sample not to be representative of our population due to study design. Importantly, being sdLDL an important atherogenic particle, the sdLDL-C percentiles were the first to be established for a European population. The newly reference intervals can now be used in patient care and an electronic application will be developed, and made available at the INSA website, for the direct determination of patient percentile for each biomarker. Interestingly, values for the P50th for TC and LDL-C are similar to the ESC/EAS recommended values, while TG P50th is the most different (Catapano et al., 2016), which is not surprisingly since TG concentration was very variable in e_COR population, probable because is very depended on diet. For the majority of the remaining parameters there are no consensus reference values for evaluation. The P50th can be considered optimal and/or a range between P25th and P75th could be adopted as reference. Above the P90th for TC, LDL-C, TG, apoB, sdLDL-C, Lp(a), non-HDL-C and remnant cholesterol, or below the P10th for HDL and apoA1 can be considered risk, and so it can be defined as the cut off for the different lipid disorders. High risk can be defined above the P95th or below the P5th. The estimated percentiles were then compared with the percentiles from other studies or populations, namely PHC (Portuguese primary health care users)

(Cortez-Dias et al., 2013), DRECE Study (Spanish) (Gómez-Gerique et al., 1999), and Framingham Offspring Study (Contois et al., 1996) and the NHANES III Study (Americans) (Bachorik et al., 1997), by plotting the percentile graphs from each study together with the estimated percentiles and corresponding estimated 95% CI from the e_COR Study, by gender and age group. A very graphical method was used to allow a good visualization of differences and similarities, being our study the first to compare the lipid percentiles among populations using this methodology. Comparison analysis showed relevant differences, even between e_COR and the other Portuguese population from PHC Study, probably due to differences in strategy design, laboratory methods and populations. Modifications of environmental factors and modulation by other non-genetic and genetic factors might also be involved, contributing for the differences at some level. In contrast, no marked differences were observed for the LDL-C and apoB percentiles between e_COR and the Framingham Offspring populations, but differences in the methodologies, as inclusion or not of medicated individuals for percentiles calculation, have to be taken in consideration.

The estimation of population specific reference values is important for the definition of optimal and at risk values. Percentiles and their confidence intervals estimation by bootstrapping is a valid way to establish reference values, especially in the case of not representative data, hence the strategy used in this study is highly recommended. It was also recommend the newly determined lipid percentiles of the Portuguese population to be used in a clinical context, since they were obtained by a rigorous and powerful methodology, so they can be used in clinical practice for patient identification and management**.**

The characterisation of dyslipidaemia patterns based on the newly determined percentiles was another of the major contributions of this work for public health[2]. The last national study for general population was in 2001 (Instituto de Alimentação Becel, 2002), so we had a gap of more than ten years without data on dyslipidaemia prevalence. Prevalence for the different lipid metabolism biomarkers showed high prevalence of severe dyslipidaemia, even though the use of statins use has increased exponentially in the last years (CEFAR, 2013). Hypercholesterolaemia prevalence was shown to be higher in women in this study, being these results different from other studies (Instituto de Alimentação Becel, 2002; Cortez-Dias et al., 2013). This must be due to the use of population specific percentiles for age and gender, revealing that dyslipidaemia in under-diagnosed in women, so population-specific reference values by gender and age are relevant, and a special attention must be drawn to dyslipidaemia in women.

It is also important to note that determination of the atherosclerotic risk based on the measurement of the most atherogenic particles was never performed in our population, and is an important issue to take in consideration when developing preventive strategies for CVD. In fact, apoB and non-HDL-C are good markers for the cardiovascular risk evaluation and are considered as secondary targeted by European guidelines (Catapano et al., 2016), since they are a measure of the concentration of the total atherogenic lipoproteins in plasma (Ramjee et al., 2011; Pencina et al., 2015). While non-HDL-C can be easily calculated without extra cost, apoB adds an additional costs to the basic lipid profile usually prescribed by the clinician. However, apolipoproteins do not require fasting conditions and are not influenced by high TG levels, which represents an advantage compared to traditional biomarkers. For instance, apoB/apoA1 ratio has been proposed as another good biomarker for predicting cardiovascular risk (Yusuf et al., 2004), where can be used as alternatives to non-HDL-C and HDL-C, respectively. Despite apoB/apoA1 ratio is not recommended as treatment target, its CVD-associated risk has been already described (Walldius et al., 2001, 2006). In the e_COR Portuguese population, findings for the prevalence of individuals not following recommendation values for apoB/apoA1 was statistically significant different from those described values, mainly because of the women's cut-off point is lower. The atherogenic risk can be also evaluated by the sdLDL-C, which is very correlated with the apoB levels, as demonstrated in this present study. Indeed, in cases where LDL-C is normal or low, high apoB levels may indicate an increased number of atherogenic particles as sdLDL-C, especially in those presenting high TG or diabetes, as was also demonstrated here.

Accordingly to the results, apoB and sdLDL-C present a very similar profile, but not when analysing values between medicated and not medicated individuals where the sdLDL-C group did not showed statistically significant difference between them, suggesting that cholesterol lowering drugs may do not decrease sdLDL in the circulation. However, the effect of statins on sdLDL particles had controversial results, so the interpretation of ours results should be done carefully (Bredie et al., 1995; Kontopoulos et al., 1996; März et al., 2001; Rosenson, 2002; Tilly-Kiesi et al., 2004; Baldassarre et al., 2005; Tokuno et al., 2007; Sniderman, 2008; Florentin et al., 2011; Yoshino et al., 2012; Diffenderfer and Schaefer, 2014; Nishikido et al., 2016). Therefore, we believe that apoB and sdLDL-C measurement will provide valuable information regarding the lipoproteins abnormalities and atherogenic risk, rather than provided by the LDL-C alone. For the dyslipidaemia characterisation of the general Portuguese population, individuals in the extreme high and low percentiles for lipids and lipoproteins were also investigated for possible monogenic causes for their phenotype. In fact, the

investigation of new variants in individuals with extremely low or high plasma lipid levels has been successfully in previously studies (Cohen et al., 2004; Patel et al., 2016; Peloso et al., 2016). In this study, were found three individuals with monogenic FH, but any other monogenic cause in the remaining individuals was found, possible due to the mild phenotype found in the extreme percentiles of the e_COR population. However, heterozygote variants, ten missenses in *PCSK9*, *APOB*, *ABCG8*, *LCAT*, *ANGPTL3*, *APOC2*, and *LPL* genes and one splice site variants in *LIPA* gene previously described as having association with lipid metabolism, were identified and might contribute to the dyslipidaemia in those individuals.

Results provide evidence on the prevalence of the different lipid disorders and reference values for each lipid biomarkers that should be taken into consideration when developing new health politics. Lp(a) and remnant cholesterol were not explored in this study, but considering that they have been suggested as important biomarkers for the CVD risk assessment, they should be analysed with more details in future studies. Although high values of dyslipidaemia have been found, dyslipidaemia is a modifiable cardiovascular risk factor, so changes in life styles and health policies must be made. Some individuals were found having monogenic FH that is associated with an elevated CVD risk *per se*, but the implementation of lipid-lowering therapy will most probably reduce their CVD-associated risk. On the other hand, dyslipidaemia can result from the influence of multiple genes with small effect, like polygenic dyslipidaemias, which are more easily modulated by modification of the life habits, and usually the implementation of lipid-lowering therapy should not be necessary. These results evidence the importance of the evaluation of dyslipidaemia aetiology that can then influence the adequate treatment for each case for a more personalised medicine and best patient prognosis.

Finally, since FH is the most common monogenic dyslipidaemia, the Portuguese cohort with a clinical diagnosis of FH was investigated. Since it is known that the FH phenotype can be associated with other monogenic disorders, the origin of the FH phenotype was explored in our cohort of patients with clinical diagnosis of FH, trough the analysis from the past 17 years of the Portuguese FH Study. All results of the Portuguese FH Study from 1999 to 2016 were assessed, and all patients were stratified into monogenic, polygenic and no apparent genetic cause for dyslipidaemia, for both children and adults index cases. For that, the LDL-C GRS using a 6 LDL-C-associated SNPs previously determined in a UK study (Talmud et al., 2013; Futema et al., 2015) was validated here, and its applicability was explored for the polygenic dyslipidaemia characterisation in the Portuguese population, while at the same type results using

different clinical FH criteria, the DLCN and SB criteria, were compared in a adult cohort. Together, were identified 39.53% (n=289) index cases with monogenic dyslipidaemia, and 13.68% (n=100) with high LDL-C GRS (above the P75th), suggesting they can have polygenic hypercholesterolaemia. This means that in 53.21% (n=389) the cause of hypercholesterolaemia was identified. If all VUS that were also identified (n=44; 6.02%) are indeed pathogenic, the total number of positives will increase to 45.55%. In the remaining 298 FH/M-, about 9.39% (n=28) presented a low LDL-C GRS (below the P25th) and could have other known or unknown cause of monogenic dyslipidaemia. However, thinking in a future perspective, for the paediatric cohort the LDL-C GRS should be validated in a children control population to confirm our results. When comparing results between DLCN and SB criteria in an adult cohort, no significant differences were found, suggesting that they probably would not have significant different discriminative power.

While monogenic dyslipidaemias are associated with an elevated CVD risk *per se*, like FH, mostly mild to severe dyslipidaemias are due to polygenic hypercholesterolaemias, as result of various genetic alterations that may interact, being modulated by non-genetic factors. It has been demonstrated that FH patients can have their life expectancy increased if early identified and treated correctly, so the correct identification of the origin of the dyslipidaemia is important for patient management including the implementation of the best therapeutic measures for the best patient prognosis. It is recommended that the genetic test for clinical FH patients should include all the monogenic dyslipidaemia-associated genes present here for the most effective patient diagnosis. Investigation on other genes causing the FH phenotype should be encouraged. For this purpose, the Portuguese FH Study will continue to investigate the genetic complexity of FH, as well as other monogenic dyslipidaemia and polygenic hypercholesterolaemia, and several NGS panels are under optimisation.

This study is the most complete biochemical and molecular characterisation of the dyslipidaemia in the Portuguese population (Figure 1). Altogether, our results advocate an urgent evaluation of dyslipidaemia aetiology and a adequate treatment for each case for a more personalised medicine and best patient prognosis, highlighting that the correct and early identification of dyslipidaemia is important for a better patient management, and could contribute to CVD prevention.

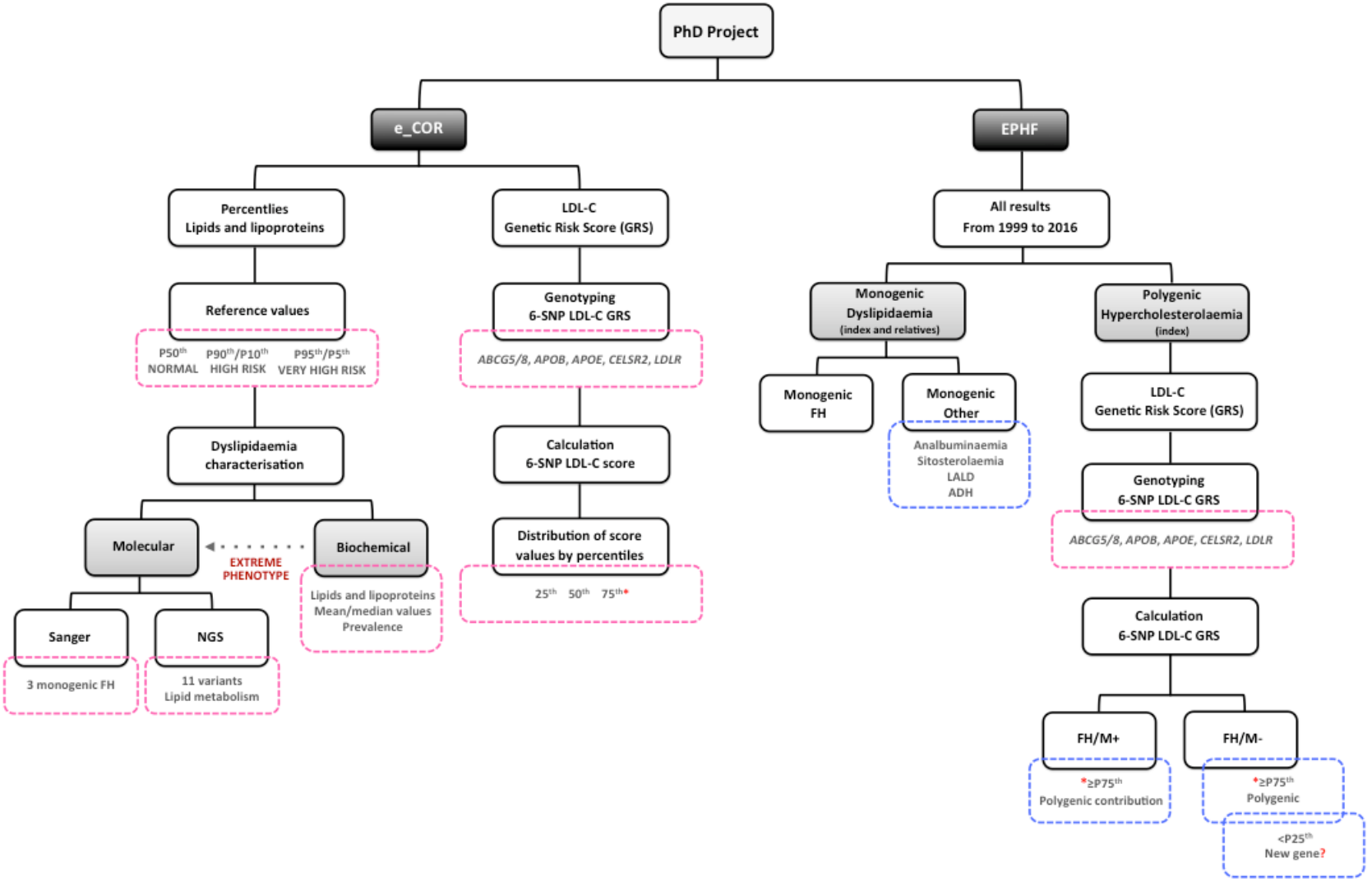

Figure 1 – Schematic representation of the present PhD project. This schematic representation summarises all work developed in this present study. FH, Familial Hypercholesterolaemia; EPHF, Portuguese FH Study; GRS; genetic risk score; P, percentile; LDL-C, low-density lipoprotein cholesterol; SNP, single nucleotide polymorphism; ADH, autosomal dominat hypercholesterolaemia; LALD, lysosomal acid lipase deficiency; NGS, next-generation sequencing.

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APPENDICES

APPENDICES

1. Appendix Tables

1.1. Appendix Table 1

Appendix Table 1 – Demographic composition of the adult population resident in Portugal in 2011.

		Number of individuals in each stratum					
Gender	**Age group**	**Norte**	**Centro**	**Lisboa**	**Alentejo**	**Algarve**	**Mainland**
Men	18-29	267,008	152,718	194,686	49,090	30,039	693,541
	30-39	271,411	159,211	222,217	52,217	34,609	739,665
	40-49	2731,39	160,439	189,442	51,837	32,193	707,050
	50-59	240,279	150,916	166,997	49,901	29,284	637,377
	60-69	183,420	130,184	151,121	41,747	24,889	531,361
	70-79	125,413	105,513	100,198	38,099	18,402	387,625
	18-79	1,360,670	858,981	1,024,661	282,891	169,416	3,696,619
Women	18-29	267,286	150,955	198,643	46,303	30,147	693,334
	30-39	286,438	166,255	235,847	51,664	35,561	775,765
	40-49	293,876	170,190	207,527	52,473	33,569	757,635
	50-59	262,511	161,153	194,239	50,589	30,274	698,766
	60-69	209,298	149,291	177,255	47,969	26,052	609,865
	70-79	166,118	134,738	1334,04	48,857	21,615	504,732
	18-79	1,485,527	932,582	1,146,915	297,855	177,218	4,040,097
Total	18-79	2,846,197	1,791,563	2,171,576	580,746	346,634	7,736,716

1.2. Appendix Table 2

Appendix Table 2 – Stratum weights used in stratified random sampling techniques.

		Stratum weights					
Gender	**Age group**	**Norte**	**Centro**	**Lisboa**	**Alentejo**	**Algarve**	**Mainland**
	18-29	0.196	0.178	0.190	0.174	0.178	0.500
	30-39	0.199	0.185	0.217	0.185	0.204	0.488
	40-49	0.201	0.187	0.185	0.183	0.190	0.483
Men	50-59	0.177	0.176	0.163	0.176	0.172	0.477
	60-69	0.135	0.152	0.148	0.148	0.147	0.466
	70-79	0.092	0.123	0.098	0.135	0.109	0.434
	18-79	0.478	0.479	0.472	0.487	0.489	0.478
	18-29	0.180	0.162	0.173	0.155	0.170	0.500
	30-39	0.193	0.178	0.206	0.173	0. 201	0.512
	40-49	0.198	0.182	0.181	0.176	0.189	0.517
Women	50-59	0.177	0.173	0.169	0.170	0.171	0.523
	60-69	0.141	0.160	0.155	0.161	0.147	0.534
	70-79	0.112	0.144	0.116	0.164	0.122	0.566
	18-79	0.522	0.521	0.528	0.513	0.511	0.522
Total	18-79	0.368	0.232	0.280	0.075	0.045	1.00

1.3. Appendix Table 3

Appendix Table 3 – Description of the variables used in data analysis, from e_COR Study.

Variables			
Name	**Description**	**Values/Units**	**Type**
NPart	Number of identification	Text	Nominal
NomeInic	Name (initials)	Text	Nominal
regiao	Region where lives	1= Norte; 2= Centro; 3= Lisboa; 4= Alentejo; 5= Algarve	Nominal
DataNasc	Birth date	Text	Nominal
Idade	Age mentioned	Years	Continuous
sexo	Gender	1= Man; 2= Woman	Nominal
PartLocalidade	City birth	Text	Nominal
PaisPart	Country of birth	1= Portugal; 2= Other	Nominal
PaisPart_outro	Other country of birth	Text	Nominal
Raca	Race	1= Caucasian; 2= African; 3= Asian; 4= Mixed; 5= Other	Nominal
Raca_outra	Other race	Text	Nominal
Medicacao_Hipertensores	Medicated for hypertension	0= No; 1= Yes	Nominal
Medicacao_Colesterol	Medicated for high cholesterol	0= No; 1= Yes	Nominal
Medicacao_Trigliceridos	Medicated for high triglycerides	0= No; 1= Yes	Nominal
Medicacao_Diabetes	Medicated for diabetes	0= No; 1= Yes	Nominal
Diabetes	Diagnosed with diabetes	1= Yes; 2= No; 3= NA	Nominal
TipoDiabetes	Type of diabetes	1= Type I; 2= Type II; 3= Gestational; 4= Mody; 5= Other	Nominal
OutroTipoDiab	Other type of diabetes	Text	Nominal
Diabetic	Diabetic[a]	0= No; 1= Yes	Nominal
EAM_estado	Myocardial infarction event	0= No; 1= Diagnosed; 2= Possible; 3= NA	Nominal
EAM_idade	Age of myocardial infarction event	Years	Continuous
Angina_estado	Diagnosed with angina	0= No; 1= Diagnosed; 2= Possible; 3= NA	Nominal
Angina_idade	Age of angina diagnosis	Years	Continuous
AVC_estado	Stroke event	0= No; 1= Diagnosed; 2= Possible; 3= NA	Nominal
AVC_idade	Age of stroke event occurred	Years	Continuous
AIT_estado	Transient ischemic incident	0= No; 1= Diagnosed; 2= Possible; 3= NA	Nominal

Continuation of Appendix Table 3 – Description of the variables used in data analysis, from e_COR Study.

Variables			
Name	**Description**	**Values/Units**	**Type**
AIT_idade	Age of transient ischemic incident	Years	Continuous
DAP_estado	Diagnosed with peripheral artery disease	0= No; 1= Diagnosed; 2= Possible; 3= NA	Nominal
DAP_idade	Age of peripheral artery disease diagnosis	Years	Continuous
HipoT_estado	Hypothyroidism	0= No; 1= Diagnosed; 2= Possible; 3= NA	Nominal
HipoT_idade	Age of hypothyroidism diagnosis	Years	Continuous
HiperT_estado	Hyperthyroidism	0= No; 1= Diagnosed; 2= Possible; 3= NA	Nominal
HiperT_idade	Age of hyperthyroidism diagnosis	Years	Continuous
Cateterismo_estado	Percutaneous transluminal coronary angioplasty	0= No; 1= Diagnosed; 2= Possible; 3= NA	Nominal
Cateterismo_idade	Age of percutaneous transluminal coronary angioplasty	Years	Continuous
Bypass_estado	Coronary artery bypass grafting	0= No; 1= Diagnosed; 2= Possible; 3= NA	Nominal
Bypass_idade	Age of coronary artery bypass grafting	Years	Continuous
HTA_ant	Family history of hypertension	0= No; 1= Yes	Nominal
CT_ant	Family history of hypercholesterolaemia	0= No; 1= Yes	Nominal
TG_ant	Family history of hypertriglyceridaemia	0= No; 1= Yes	Nominal
Diab_ant	Family history of diabetes	0= No; 1= Yes	Nominal
EAM_ant	Family history of premature myocardial infarction	0= No; 1= Yes	Nominal
DCV_ant	Family history of coronary heart disease	0= No; 1= Yes	Nominal
EAM_prematuro	Family history of premature myocardial infarction[a]	0= No; 1= Yes	Nominal
DCV_prematura	Family history of premature coronary heart disease[a]	0= No; 1= Yes	Nominal
Fumador	Smoking habits	0= No; 1= Yes	Nominal
N_Fumo	Number of cigarettes per day	Unit	Continuous
Dieta_equilibrada	Balanced diet[a]	0= No; 1= Yes	Nominal
Unid_dia	Alcohol intake per day	Unit	Continuous
IPAQ	Physical activity according to IPAQ	1= High level; 2= Moderate level; 3= Low level	Nominal
TempoSentadoSemanal	Hours sitting per day	Hours	Continuous
CHormonais	Hormonal user	0= No; 1= Yes	Nominal
Glicose	Biochemical determination of glucose	mg/dL	Continuous
ColesterolTotal	Biochemical determination of total cholesterol	mg/dL	Continuous

Continuation of Appendix Table 3 – Description of the variables used in data analysis, from e_COR Study.

Variables			
Name	**Description**	**Values/Units**	**Type**
HDL_colheita	Biochemical determination of HDL-C	mg/dL	Continuous
LDL_colheita	Biochemical determination of LDL-C	mg/dL	Continuous
Lpa	Biochemical determination of lipoprotein (a)	mg/dL	Continuous
ApoA1	Biochemical determination of apolipoprotein A1	mg/dL	Continuous
ApoB	Biochemical determination of apolipoprotein B	mg/dL	Continuous
TG	Biochemical determination of triglycerides	mg/dL	Continuous
AST_GOT	Biochemical determination of aspartate aminotransferase	mg/dL	Continuous
ALT_GPT	Biochemical determination of alanine aminotransferase	mg/dL	Continuous
GGT	Biochemical determination of gamma-glutamyl transpeptidase	mg/dL	Continuous
sdLDL_Daytona	Biochemical determination of triglycerides	mg/dL	Continuous
PAS	Systolic blood pressure	mmHg	Continuous
PAD	Diastolic blood pressure	mmHg	Continuous
HTA	Hypertension[a]	0= No; 1= Yes	Nominal
Peso	Weight	Kilograms (kg)	Continuous
Altura	Height	Centimeters (cm)	Continuous
IMC	Body mass index	kg/m^2	Continuous
IMC_classes	Body mass index classification[a]	1= Underweight; 2= Normal; 3= Overweight; 4= Obesity grade I; 5= Obesity grade II; 6= Obesity grade III; 7= ND	Nominal

NA, no answer; ND, not determined; IPAQ, International Physical Activity Questionnaire.

[a]Diabetes, premature cardiovascular disease, balanced diet, hypertension, and overweight/obesity were classified according to the national and/or international guidelines and recommendations, as described in the Supplementary Material section of Chapter 3 of this study.

1.4. Appendix Table 4

Appendix Table 4 – Description of the variables created for data analysis, from e_COR Study.

Variables			
Name	**Description**	**Values/Units**	**Type**
apb_apa	Apolipoprotein B/apolipoprotein A1 ratio	mg/dL	Continuous
non_HDL	Non-HDL-C	mg/dL	Continuous
tc_cor	Total cholesterol after correction for statins[b]	mg/dL	Continuous
ldl_cor	LDL-C after correction for statins[b]	mg/dL	Continuous
apob_cor	Apolipoprotein B after correction for statins[b]	mg/dL	Continuous
nhdl_cor	Non-HDL-C after correction for statins for total cholesterol[b]	mg/dL	Continuous
sd_ldl	Small dense LDL-C/LDL-C ratio	mg/dL	Continuous
sd_ldl_cor	Small dense LDL-C/LDL-C ratio after correction for statins for LDL-C[b]	mg/dL	Continuous
g_L	Alcohol intake per day	g/L	Continuous
bmi_var	Normal weight. overweight or obesity[a]	1= Normal; 2= Overweight; 3= Obesity	Nominal
hta_var	Normal. prehypertension or hypertension[a]	1= Normal; 2= Prehypertension; 2= Hypertension	Nominal
diab_var	Normal. pre-diabetes or diabetes[a]	1= Normal; 2= Pre-diabetes; 2= Diabetes	Nominal
hypot_cat	Diagnosed with hypothyroidism	0= No; 1= Yes	Nominal
hypert_cat	Diagnosed with hyperthyroidism	0= No; 1= Yes	Nominal
alcohol_cat	Moderate consumption according to the guidelines recommendations[a]	0= No; 1= Yes	Nominal
p1apob	Below 5th percentile for apolipoprotein B after correction for statins[b]	0= No; 1= Yes	Nominal
p2apob	Below 50th percentile for apolipoprotein B after correction for statins[b]	0= No; 1= Yes	Nominal
p3apob	Above 90th percentile for apolipoprotein B after correction for statins[b]	0= No; 1= Yes	Nominal
p4apob	Above 95th percentile for apolipoprotein B after correction for statins[b]	0= No; 1= Yes	Nominal
p50apob	Below 50th percentile for apolipoprotein B after treatment[c]	0= No; 1= Yes	Nominal
p90apob	Above 90th percentile for apolipoprotein B after treatment[c]	0= No; 1= Yes	Nominal
p95apob	Above 95th percentile for apolipoprotein B after treatment[c]	0= No; 1= Yes	Nominal
p1tc	Below 5th percentile for total cholesterol after correction for statins[b]	0= No; 1= Yes	Nominal
p2tc	Below 50th percentile for total cholesterol after correction for statins[b]	0= No; 1= Yes	Nominal
p3tc	Above 90th percentile for total cholesterol after correction for statins[b]	0= No; 1= Yes	Nominal

Continuation of Appendix Table 4 – Description of the variables created for data analysis, from e_COR Study.

Variables			
Name	**Description**	**Values/Units**	**Type**
p4tc	Above 95th percentile for total cholesterol after correction for statins[b]	0= No; 1= Yes	Nominal
p50tc	Below 50th percentile for total cholesterol after treatment[c]	0= No; 1= Yes	Nominal
p90tc	Above 90th percentile for total cholesterol after treatment[c]	0= No; 1= Yes	Nominal
p95tc	Above 95th percentile for total cholesterol after treatment[c]	0= No; 1= Yes	Nominal
p1ldl	Below 5th percentile for LDL-C after correction for statins[b]	0= No; 1= Yes	Nominal
p2ldl	Below 50th percentile for LDL-C after correction for statins[b]	0= No; 1= Yes	Nominal
p3ldl	Above 90th percentile for LDL-C after correction for statins[b]	0= No; 1= Yes	Nominal
p4ldl	Above 95th percentile for LDL-C after correction for statins[b]	0= No; 1= Yes	Nominal
p50ldl	Below 50th percentile for LDL-C after treatment[c]	0= No; 1= Yes	Nominal
p90ldl	Above 90th percentile for LDL-C after treatment[c]	0= No; 1= Yes	Nominal
p95ldl	Above 95th percentile for LDL-C after treatment[c]	0= No; 1= Yes	Nominal
p1hdl	Below 5th percentile for HDL-C	0= No; 1= Yes	Nominal
p2hdl	Above 50th percentile and below 90th percentile for HDL-C	0= No; 1= Yes	Nominal
p3hdl	Above 50th percentile and below 95th percentile for HDL-C	0= No; 1= Yes	Nominal
p4hdl	Above 90th percentile for HDL-C	0= No; 1= Yes	Nominal
p5hdl	Above 95th percentile for HDL-C	0= No; 1= Yes	Nominal
p6hdl	Below 10th percentile for HDL-C	0= No; 1= Yes	Nominal
p1apoa1	Below 5th percentile for apolipoprotein A1	0= No; 1= Yes	Nominal
p2apoa1	Above 50th percentile and below 90th percentile for apolipoprotein A1	0= No; 1= Yes	Nominal
p3apoa1	Above 50th percentile and below 95th percentile for apolipoprotein A1	0= No; 1= Yes	Nominal
p4apoa1	Above 90th percentile for apolipoprotein A1	0= No; 1= Yes	Nominal
p5apoa1	Above 95th percentile for apolipoprotein A1	0= No; 1= Yes	Nominal
p6apoa1	Below 10th percentile for apolipoprotein A1	0= No; 1= Yes	Nominal
p1nhdl	Below 5th percentile for non-HDL-C after correction for statins[b]	0= No; 1= Yes	Nominal
p2nhdl	Below 50th percentile for non-HDL-C after correction for statins[b]	0= No; 1= Yes	Nominal
p3nhdl	Above 90th percentile for non-HDL-C after correction for statins[b]	0= No; 1= Yes	Nominal
p4nhdl	Above 95th percentile for non-HDL-C after correction for statins[b]	0= No; 1= Yes	Nominal
p1sdldl	Below 5th percentile for small dense LDL-C	0= No; 1= Yes	Nominal
p2sdldl	Above 50th percentile and below 90th percentile for small dense LDL-C	0= No; 1= Yes	Nominal
p3sdldl	Above 50th percentile and below 95th percentile for small dense LDL-C	0= No; 1= Yes	Nominal

Continuation of Appendix Table 4 – Description of the variables created for data analysis, from e_COR Study.

Variables			
Name	**Description**	**Values/Units**	**Type**
p4sdldl	Above 90th percentile for small dense LDL-C	0= No; 1= Yes	Nominal
p5sdldl	Above 95th percentile for small dense LDL-C	0= No; 1= Yes	Nominal
p1tg	Below 5th percentile for small dense LDL-C	0= No; 1= Yes	Nominal
p2tg	Above 50th percentile and below 90th percentile for triglycerides	0= No; 1= Yes	Nominal
p3tg	Above 50th percentile and below 95th percentile for triglycerides	0= No; 1= Yes	Nominal
p4tg	Above 90th percentile for small dense triglycerides	0= No; 1= Yes	Nominal
p5tg	Above 95th percentile for small dense triglycerides	0= No; 1= Yes	Nominal
snp1	rs10401969 (CC, CT, TT)	1= CC; 2= CT; 3= TT	Nominal
snp2	rs12027135 (AA, AT, TT)	1= AA; 2= AT; 3= TT	Nominal
snp3	rs1260326 (CC, CT, TT)	1= CC; 2= CT; 3= TT	Nominal
snp4	rs12678919 (AA, AG, GG)	1= AA; 2= AG; 3= GG	Nominal
snp5	rs12740374 (GG, GT, TT)	1= GG; 2= GT; 3= TT	Nominal
snp6	rs12916 (CC, CT, TT)	1= CC; 2= CT; 3= TT	Nominal
snp7	rs1367117 (AA, AG, GG)	1= AA; 2= AG; 3= GG	Nominal
snp8	rs1532085 (AA, AG, GG)	1= AA; 2= AG; 3= GG	Nominal
snp9	rs1564348 (CC, CT, TT)	1= CC; 2= CT; 3= TT	Nominal
snp10	rs16942887 (AA, AG, GG)	1= AA; 2= AG; 3= GG	Nominal
snp11	rs17145738 (CC, CT, TT)	1= CC; 2= CT; 3= TT	Nominal
snp12	rs174546 (CC, CT, TT)	1= CC; 2= CT; 3= TT	Nominal
snp13	rs1883025 (CC, CT, TT)	1= CC; 2= CT; 3= TT	Nominal
snp14	rs2000999 (AA, AG, GG)	1= AA; 2= AG; 3= GG	Nominal
snp15	rs2131925 (GG, GT, TT)	1= GG; 2= GT; 3= TT	Nominal
snp16	rs2479409 (AA, AG, GG)	1= AA; 2= AG; 3= GG	Nominal
snp17	rs2954029 (AA, AT, TT)	1= AA; 2= AT; 3= TT	Nominal
snp18	rs3757354 (CC, CT, TT)	1= CC; 2= CT; 3= TT	Nominal
snp19	rs3764261 (AA, AC, CC)	1=AA; 2= AC; 3= CC	Nominal
snp20	rs429358 (CC, CT, TT)	1= CC; 2= CT; 3= TT	Nominal
snp21	rs4299376 (GG, GT, TT)	1= GG; 2= GT; 3= TT	Nominal
snp22	rs4420638 (AA, AG, GG)	1= AA; 2= AG; 3= GG	Nominal
snp23	rs4846914 (AA, AG, GG)	1= AA; 2= AG; 3= GG	Nominal
snp24	rs629301 (GG, GT, TT)	1= GG; 2= GT; 3= TT	Nominal

Continuation of Appendix Table 4 – Description of the variables created for data analysis, from e_COR Study.

Variables			
Name	**Description**	**Values/Units**	**Type**
snp25	rs635634 (CC, CT, TT)	1= CC; 2= CT; 3= TT	Nominal
snp26	rs6511720 (GG, GT, TT)	1= GG; 2= GT; 3= TT	Nominal
snp27	rs6882076 (CC, CT, TT)	1= CC; 2= CT; 3= TT	Nominal
snp28	rs7241918 (GG, GT, TT)	1= GG; 2= GT; 3= TT	Nominal
snp29	rs7412 (CC, CT, TT)	1= CC; 2= CT; 3= TT	Nominal
snp30	rs964184 (CC, CG. GG)	1= CC; 2= CG; 3= GG	Nominal
snp31	rs9987289 (AA, AG, GG)	1= AA; 2= AG; 3= GG	Nominal
snp32	rs6065906 (CC, CT, TT)	1= CC; 2= CT; 3= TT	Nominal
apoe_h2	*APOE* gene haplotype[d]	1= E2E2; 2= E2E3; 3= E2E4; 4= E3E3; 5= E3E4; 6= E4E4	Nominal
ldl_score	Sum of the effect sizes of 6 SNPs of the LDL-C gene score[e]	mmol/L	Continuous
scoreldl	Has LDL-C score value	0= No; 1= Yes	Nominal
hdl_score	Sum of the effect sizes of lead SNPs for HDL-C[f]	Unit	Continuous
scorehdl	Has HDL-C score value	0= No; 1= Yes	Nominal
tg_score	Sum of the effect sizes of lead SNPs for triglycerides[f]	Unit	Continuous
scoretg	Has triglycerides score value	0= No; 1= Yes	Nominal
quar_ldl	LDL-C gene score by percentiles	1= 25th; 2= 50th; 3= 75th; 4= 100th	Nominal
quar_hdl	Sum of the effect sizes of lead SNPs for HDL-C by quartiles	1=Q1; 2= IQR; 3= Q3	Nominal
quar_tg	Sum of the effect sizes of lead SNPs for triglycerides by quartiles	1=Q1; 2= IQR; 3= Q3	Nominal
sum_ldl	Sum of polymorphisms for LDL-C	Unit	Continuous
sum_hdl	Sum of polymorphisms for HDL-C	Unit	Continuous
sum_tg	Sum of polymorphisms for triglycerides	Unit	Continuous

SNP, single nucleotide polymorphism.

[a]Overweight/obesity, hypertension, diabetes,and type of alcohol intake were classified according to the national and/or international guidelines and recommendations, as described in the Supplementary Material section of Chapter of this study.

[b]Correction factors were used to estimate untreated values for total cholesterol, LDL-C, and apoB, as described in the Methods section of Chapter of this study.

[C]Once identified as under lipid-lowering therapy with statins, values for TC, LDL-C, and apoB were considered as measured values after treatment.

[d]The *APOE* SNPs (rs7412 and rs429358) determined the *APOE* haplotype, an important genetic determinant of LDL-C levels, by resulting in different isoforms of the apolipoprotein E (ApoE): ε2, ε3, and ε4.

[e]The LDL-C genetic risk score was determined as previously reported by Talmud, Futema and colleagues's study, as described in the Methods section of Chapter 4 of this study.

[f]The effect size values of the lead SNPs for HDL-C and triglycerides were taken from Teslovich and colleagues' study, as described in the Methods section of Chapter of this study.

1.5. Appendix Table 5

Appendix Table 5 – Description of the variables created for data analysis from Portuguese FH Study.

Variables			
Name	**Description**	**Values/Units**	**Type**
consider	Group to consider in data analysis	1= Out; 2= Other; 3= 2017; 4= Negative; 5= Positive	Nominal
vus	Variant of uncertain significance	0= No; 1= Yes	Nominal
family	Number of the family in the Portuguese FH Study	Text	Nominal
id	Number of identification in the Portuguese FH Study	Text	Nominal
index_relative	Index or relative cases	1= Index; 2= Relative	Nominal
age	Age at time of their inclusion in the Portuguese FH Study	Years	Continuous
gender	Man or woman	1= Man; 2= Woman	Nominal
bmi	Body mass index	kg/m^2	Continuous
bmi_25	Body mass index above 25 kg/m^{2a}	0= No; 1= Yes	Nominal
bmi_percentile	Body mass index percentile in children [a]	1= 5th; 2= 5th-10th; 3= 10th-25th; 4= 25th-50th; 5= 50th-75th; 6= 75th-85th; 8= 85th-95th; 9= 95th	Nominal
smoking	Smoking habits	0= No; 1= Yes	Nominal
cigarettes_day	Number of cigarettes per day	Unit	Continuous
alcohol_use	Alcohol consumption	0= No; 1= Yes	Nominal
Unitss_week	Alcohol intake per week	g/L	Continuous
exercising	Physical activity	0= No; 1= Yes	Nominal
diet	Under diet	0= No; 1= Yes	Nominal
statins	Under lipid-lowering therapy with statins	0= No; 1= Yes	Nominal
hypertension	Hypertension	0= No; 1= Yes	Nominal
diabetes	Diagnosed with diabetes	0= No; 1= Yes	Nominal
angina	Diagnosed with angina	0= No; 1= Yes	Nominal
angina_age	Age of angina diagnosis	Years	Continuous
MI	Myocardial infarction event	0= No; 1= Yes	Nominal
MI_age	Age of myocardial infarction event ocurred	Years	Continuous
ptca	Percutaneous transluminal coronary angioplasty	0= No; 1= Yes	Nominal
ptca_age	Age of percutaneous transluminal coronary angioplasty	Years	Continuous
cabg	Coronary artery bypass grafting	0= No; 1= Yes	Nominal
cabg_age	Age of coronary artery bypass grafting	Years	Continuous

Continuation of Appendix Table 5 – Description of the variables created for data analysis, from Portuguese FH Study.

Variables			
Name	**Description**	**Values/Units**	**Type**
stroke	Stroke event	0= No; 1= Yes	Nominal
stroke_age	Age of stroke event occurred	Years	Continuous
other_CVD	Other cardiovascular disease	Text	Nominal
CVD	Cardiovascular disease event	0= No; 1= Yes	Nominal
pCVD	Premature cardiovascular disease event (before age 55 in men and 65 in women)[a]	0= No; 1= Yes	Nominal
corneal_arcus	Corneal arcus xanthoma	0= No; 1= Yes	Nominal
xanthelasma	Xanthelasma	0= No; 1= Yes	Nominal
back_hands	Back hands xanthoma	0= No; 1= Yes	Nominal
pretibial	Pretibial xanthoma	0= No; 1= Yes	Nominal
elbows	Elbows xanthoma	0= No; 1= Yes	Nominal
achilles_tendon	Achilles tendon xanthoma	0= No; 1= Yes	Nominal
tc_1	Total cholesterol mentioned by the physician	mg/dL	Continuous
ldl_1	LDL-C mentioned by the physician	mg/dL	Continuous
hdl_1	HDL-C mentioned by the physician	mg/dL	Continuous
tg_1	Triglycerides mentioned by the physician	mg/dL	Continuous
non_hdl_1	Non-HDL-C calculated with values mentioned by physician	mg/dL	Continuous
tc_2	Biochemical determination of total cholesterol in INSA	mg/dL	Continuous
ldl_2	Biochemical determination of LDL-C in INSA	mg/dL	Continuous
hdl_2	Biochemical determination of HDL-C in INSA	mg/dL	Continuous
tg_2	Biochemical determination of triglycerides in INSA	mg/dL	Continuous
lpa_2	Biochemical determination of lipoprotein (a) in INSA	mg/dL	Continuous
apoa1_2	Biochemical determination of apolipoprotein A1 in INSA	mg/dL	Continuous
apob_2	Biochemical determination ofapolipoprotein B in INSA	mg/dL	Continuous
apob_4	Apolipoprotein B after correction for statins[b]	mg/dL	Continuous
apob_apoa1_2	Apolipoprotein B/apolipoprotein A1 ratio	mg/dL	Continuous
apob_apoa1_4	Apolipoprotein B/apolipoprotein A1 ratio after correction for statins[b]	mg/dL	Continuous
non_hdl_4	Non-HDL-C calculated biochemical values determined in INSA	mg/dL	Continuous
tc_4	Total cholesterol determined in INSA, after correction for statins[b]	mg/dL	Continuous
ldl_4	LDL-C determined in INSA, after correction for statins[b]	mg/dL	Continuous

Continuation of Appendix Table 5 – Description of the variables created for data analysis, from Portuguese FH Study.

Variables			
Name	**Description**	**Values/Units**	**Type**
non-hdl_4	Non-HDL-C calculated with total cholesterol after correction for statins[b]	mg/dL	Continuous
ldl_5	LDL-C value considered at time of their inclusion in the EPHF Study	mg/dL	Continuous
tc_5	Total cholesterol value considered at time of their inclusion in the EPHF Study	mg/dL	Continuous
hdl_5	HDL-C value considered at time of their inclusion in the EPHF Study	mg/dL	Continuous
tg_5	Triglycerides value considered at time of their inclusion in the EPHF Study	mg/dL	Continuous
apoe_hap	*APOE* gene haplotype	1= E2E2; 2= E2E3; 3= E2E4; 4= E3E3; 5= E3E4; 6= E4E4	Nominal
result_final	Final FH diagnosis	1= Positive; 2= Negative	Nominal
variant_1	Variant in FH gene	Text	Nominal
acmg	Classification of the variant (ACMG classification, Chora et al., 2017)	1= Likely benign; 2= Benign; 3= Likely pathogenic; 4= Pathogenic; 4= VUS	Nominal
variant_2	Other variant in FH gene	Text	Nominal
acmg_2	Classification of the other variant in FH gene (ACMG classification, Chora et al., 2017)	1= Likely benign; 2= Benign; 3= Likely pathogenic; 4= Pathogenic; 4= VUS	Nominal
allele_type	Allele type for *LDLR* gene variant	1= Null; 2= Defective; 3= NDA	Nominal
variant_3	Variant in other gene	Text	Nominal
ldl_score_fh	Sum of the effect sizes of 6 SNPs of the LDL-C gene score[c]	Unit	Continuous
scoreldl_fh	Has LDL-C gene score value	0= No; 1= Yes	Nominal
quarter	LDL-C value distributed by percentiles	1= 25th; 2= 50th; 3= 75th; 4= 100th	Nominal
hdl_score_fh	Sum of the effect sizes of lead SNPs for HDL-C[d]	Unit	Continuous
scorehdl_fh	Has HDL-C score value	0= No; 1= Yes	Nominal
tg_score_fh	Sum of the effect sizes of lead SNPs for triglycerides[d]	Unit	Continuous
scoretg_fh	Has triglycerides score value	0= No; 1= Yes	Nominal
quar_hdl_fh	Sum of the effect sizes of lead SNPs for HDL-C by quartiles	1= Q1; 2=IQR; 3= Q3	Nominal
quar_tg_fh	Sum of the effect sizes of lead SNPs for triglycerides by quartiles	1= Q1; 2=IQR; 3= Q3	Nominal

Continuation of Appendix Table 5 – Description of the variables created for data analysis, from Portuguese FH Study.

Variables			
Name	**Description**	**Values/Units**	**Type**
sum_hdl_fh	Sum of polymorphisms for HDL-C	Unit	Continuous
sum_tg_fh	Sum of polymorphisms for triglycerides	Unit	Continuous
null_Q1_Q4	Null alleles according to the LDL-C gene score	1= 25th; 2= 75th	Nominal
defective_Q1_Q4	Defective alleles according to the LDL-C gene score	1= 25th; 2= 75th	Nominal
null_def_Q1_Q4	Undefined allele type (null/defective) according to the LDL-C gene score	1= 25th; 2= 75th	Nominal
DLCNS1	First degree relative with known premature coronary and/or vascular disease (before age 55 in men and 60 in women)	0= No; 1= Yes	Nominal
score_1	Score (1 point)	Unit	Continuous
DLCNS2	First degree relative (adult) with known LDL-cholesterol above the 95th percentile for age and gender	0= No; 1= Yes	Nominal
score_2	Score (1 point)	Unit	Continuous
DLCNS3	First degree relative with tendinous xanthomata and/or arcus cornealis	0= No; 1= Yes	Nominal
score_3	Score (2 points)	Unit	Continuous
DLCNS4	Children aged < 18 years with LDL-cholesterol above the 95th percentile for age and gender	0= No; 1= Yes	Nominal
score_4	Score (2 points)	Unit	Continuous
DLCNS5	Patients with premature coronary artery disease (before age 55 in men and 60 in women)	0= No; 1= Yes	Nominal
score_5	Score (2 points)	Unit	Continuous
DLCNS6	Patients with premature cerebral or peripheral vascular disease (before age 55 in men and 60 in women)	0= No; 1= Yes	Nominal
score_6	Score (1 point)	Unit	Continuous
DLCNS7	Tendinous xanthomata	0= No; 1= Yes	Nominal
score_7	Score (6 point)	Unit	Continuous
DLCNS8	Arcus cornealis before 45 years of age	0= No; 1= Yes	Nominal

Continuation of Appendix Table 5 – Description of the variables created for data analysis, from Portuguese FH Study.

Variables			
Name	**Description**	**Values/Units**	**Type**
score_8	Score (4 points)	Unit	Continuous
DLCNS9	LDL-C ≥ 8.5 mmol/L	0= No; 1= Yes	Nominal
score_9	Score (8 points)	Unit	Continuous
DLCNS10	LDL-C 6.5-8.4 mmol/L	0= No; 1= Yes	Nominal
score_10	Score (5 points)	Unit	Continuous
DLCNS11	LDL-C 5.0-6.4 mmol/L	0= No; 1= Yes	Nominal
Score_11	Score (3 points)	Unit	Continuous
DLCNS12	LDL-C 4.0-4.9 mmol/L	0= No; 1= Yes	Nominal
Score_12	Score (1 point)	Unit	Continuous
SB1	Has family history of hypercholesterolaemia (first and/or second degree)	0= No; 1= Yes	Nominal
SB2	Has family history of premature cardiovascular disease (first and/or second degree)[a]	0= No; 1= Yes	Nominal
SB3	Has Tendon xanthomas in the patient or any of the patient´s first or second degree relatives	0= No; 1= Yes	Nominal
diagnosis_1	Clinical diagnosis of FH according to the Simom Broome criteria	1= Definite; 2= Probable; 3= Unlikely; 4= No information	Nominal
diagnosis_2	Clinical diagnosis of FH according to the Dutch criteria (DLCNS)	1= Definite; 2= Probable; 3= Unlikely; 4= No information	Nominal

FH, Familial Hypercholesterolaemia; EPHF, Portuguese Familial hypercholesterolaemia Study; SNP, single nucleotide polymorphism; INSA, National Institute of Health Doctor Ricardo Jorge; DLCNS, Dutch Lipid Clinic Network Score; NDA, not determined allele.

[a]Overweight/obesity, and premature cardiovascular disease were classified according to the national and/or international guidelines and recommendations, as described in the Supplementary Material section of Chapter 2 of this study.

[b]Correction factors were used to estimate untreated values for total cholesterol, LDL-C, and apoB, as described in the Methods section of Chapter 2 of this study.

[c]The LDL-C genetic risk score was determined as previously reported by Talmud et al. (2013) and Futema et al. (2015) studies, as described in the Methods section of Chapter 4 of this study.

[d]The effect size values of the lead SNPs for HDL-C and triglycerides were taken from Teslovich et al. study (2010), as described in the Methods section of Chapter 4 of this study.

1.6. Appendix Table 6

Appendix Table 6 – Description of the variables created for data analysis, from Portuguese FH Study and e_COR Study.

Variables			
Name	**Description**	**Values/Units**	**Type**
study	Which study	1= EPHF; 2= e_COR	Nominal
category	Positive. negative or e_COR	1= Positive; 2= Negative; 3= e_COR	Nominal
id_all	Number of identification in the Portuguese FH Study or e_COR Study	Text	Nominal
age_all	Age	Years	Continuous
ldl_score_all	Sum of the effect sizes of 6 SNPs of the LDL-C gene score[a]	mmol/L	Continuous
scoreldl_all	Has LDL-C score value	0= No; 1= Yes	Nominal
hdl_score_all	Sum of the effect sizes of lead SNPs for HDL-C[b]	Unit	Continuous
scorehdl_all	Has HDL-C score value	0= No; 1= Yes	Nominal
tg_score_all	Sum of the effect sizes of lead SNPs for triglycerides[b]	Unit	Continuous
scoretg_all	Has triglycerides score value	0= No; 1= Yes	Nominal
quar_ldl_all	Distribution of LDL-C gene score by percentiles	1= 25th; 2= 50th; 3= 75th	Nominal
quar_hdl_all	Distribution by percentiles of the sum of the effect sizes of lead SNPs for HDL-C	1= 25th; 2= 50th; 3= 75th	Nominal
quar_tg_all	Distribution by percentiles of the sum of the effect sizes of lead SNPs for triglycerides	1= 25th; 2= 50th; 3= 75th	Nominal
sum_ldl_all	Sum of polymorphisms for LDL-C	Unit	Continuous
sum_hdl_all	Sum of polymorphisms for HDL-C	Unit	Continuous
sum_tg_all	Sum of polymorphisms for triglycerides	Unit	Continuous

FH, familial hypercholesterolaemia; EPHF, Portuguese Familial Hypercholesterolaemia Study; SNP, single nucleotide polymorphism.

[a]The LDL-C genetic risk score was determined as previously reported by Talmud et al. (2013) and Futema et al. (2015) studies, as described in the Methods section of Chapter 4 of this study.

[b]The effect size values of the lead SNPs for HDL-C and triglycerides were taken from Teslovich et al. study (2010), as described in the Methods section of Chapter 4 of this study.